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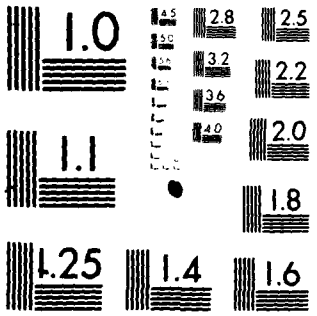

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER
Technical Report No. 4

2. GOVT ACCESSION NO.
AD-A116 259

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
Immunoregulation

5. TYPE OF REPORT & PERIOD COVERED
Interim (annual)

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Dr. Jonathan R. Lamb, Ph.D.
Dr. Philip L. Calcagno, M.D.

8. CONTRACT OR GRANT NUMBER(s)
N000-14-77-C-0748

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Immunologic Oncology Division, Dept. of Pediatrics
Lombardi Cancer Center, Georgetown University
Washington, D.C. 20007

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
[redacted]. NR 207-102
see [illegible]

11. CONTROLLING OFFICE NAME AND ADDRESS
Office of Naval Research, Biophysics (Code 444),
800 N. Quincy Street, Arlington, VA 22217

12. REPORT DATE
May 1981

13. NUMBER OF PAGES

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
See enclosed list

"A"

APPROVED FOR PUBLIC RELEASE
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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

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MAY 29 1981
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18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Immunoregulation; clonal deletion; anti-receptor antibodies; transplantation; cellular immunity lymphocytes, cell surface receptors; protective immunity.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In order to successfully treat individuals exposed to ionizing irradiation using bone marrow transplantation, ways must be developed that will allow us to regulate both recipient and donor immune responses, and to specifically delete clones of harmful cells. Under this contract, antibodies are being developed that recognize specific cell surface structures and receptors as well as cell interaction molecules. These reagents will allow specific clones, such as those involved in inducing graft versus host disease in bone marrow

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transplantation, to be eliminated, leaving intact the remainder of the immune system. Furthermore, such anti-receptor antibodies have the property of triggering certain cell subsets, and this would facilitate the manipulation of specific clones of cells required for protective immunity to infectious agents.

In our previous technical reports we have been able to show that, in animal models, antibodies can be prepared against cell receptors for histocompatibility antigens. Our current work is focused on applying these model studies to the human immune system.

Over the past year we have developed in vitro techniques for the analysis of specific human immune responses to a variety of antigens including influenza and tetanus toxoid. These in vitro models will allow us to analyze in detail the effects of anti-receptor and anti-cell interaction molecule antisera on specific immune responses. Indeed, we report the inhibitory effect of certain antisera to HLA-DR antigens on tetanus toxoid and influenza virus specific responses. In the rodent and also in the human we have analyzed the effect of anti-T cell and anti-Ia (DR) antisera on mixed lymphocyte responses, ie. responses to foreign histocompatibility antigens.

The analysis of cell interaction molecules has focused on the physico-chemical analysis of helper and suppressor factor molecules, and on the ability of antisera against cell surface structures to bind and manipulate the functional reactivity of helper factor molecules. Finally, T cells specific for HLA-D and DR antigens as well as influenza virus specific T cells have been formally cloned providing us with an expanded population of homogeneous cells with which to develop anti-idiotype reagents.

Future studies will continue with the development of biological reagents that recognize cell surface structures and cell interaction molecules, and to study the effect of these reagents on specific immune responses.

OFFICE OF NAVAL RESEARCH
Contract N000-14-77-C-0748
Task No. NR 207-102
TECHNICAL REPORT NO. 4

IMMUNO REGULATION

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24th April 1981

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A. ABSTRACT

In order to successfully treat individuals exposed to ionizing irradiation using bone marrow transplantation, ways must be developed that will allow us to regulate both recipient and donor immune responses, and to specifically delete clones of harmful cells. Under this contract, antibodies are being developed that recognize specific cell surface structures and receptors as well as cell interaction molecules. These reagents will allow specific clones, such as those involved in inducing graft versus host disease in bone marrow transplantation, to be eliminated, leaving intact the remainder of the immune system. Furthermore, such anti-receptor antibodies have the property of triggering certain cell subsets, and this would facilitate the manipulation of specific clones of cells required for protective immunity to infectious agents.

In our previous technical reports we have been able to show that, in animal models, antibodies can be prepared against cell receptors for histocompatibility antigens. Our current work is focused on applying these model studies to the human immune system.

Over the past year we have developed *in vitro* techniques for the analysis of specific human immune responses to a variety of antigens including influenza and tetanus toxoid. These *in vitro* models will allow us to analyse in detail the effects of anti-receptor and anti-cell interaction molecule antisera on specific immune responses. Indeed, we report the inhibitory effect of certain antisera to HLA-DR antigens on tetanus toxiod and influenza virus specific responses. In the rodent and also in the human we have analyzed the effect of anti-T cell and anti-Ia (DR) antisera on mixed lymphocyte responses, ie. responses to foreign histocompatibility antigens.

The analysis of cell interaction molecules has focused on the physicochemical analysis of helper and suppressor factor molecules, and on the ability of antisera against cell surface structures to bind and manipulate the functional reactivity of helper factor molecules. Finally, T cells specific for HLA-D and DR antigens as well as influenza virus specific T cells have been formally cloned providing us with an expanded population of homogeneous cells with which to develop anti-idiotype reagents.

Future studies will continue with the development of biological reagents that recognize cell surface structures and cell interaction molecules, and to study the effect of these reagents on specific immune responses.

B. INTRODUCTION

The following technical report summarizes the fourth year's progress on ONR Contract N000-14-C-0748 "Immunoregulation of facilitate transplantation and reparative surgery: development of natural biological agents" which was initiated 15 September 1977 for the purpose of developing a system for modifying immune responses without compromising the general health of the individual. The contract title was shortened to "Immuno Regulation" in 1979 to be more descriptive of the overall project.

C. BACKGROUND

Bone marrow transplantation offers the only hope to victims whose stem cells have not developed, in the case of children with immune deficiency diseases, or in cases where the stem cells have been destroyed by chemical agents or irradiation. In the civilian community bone marrow transplantation has shown considerable promise in the therapy of severe aplastic anemia, and has been used in the treatment of malignancy in particular acute leukemia.

In the case of aplastic anemia, patients receiving bone marrow from histocompatible donors show a 30 month survival of 57% opposed to a 25% survival of non-transplanted patients. From 20-40% of the grafts initially fail, usually due to patient presensitization and rejection of the grafts, as these patients have generally received many blood transfusions sensitizing them to foreign HLA antigens. A variety of complex drug regimens have developed to prepare the presensitized patient for engaftment.

The most significant complication of bone marrow transplant is the syndrome known as graft versus host disease (GVHD). The acute form of this disease is thought to be caused by immune competent T (Thymus derived) lymphocytes present in the door bone marrow cells used for engraftment. These cells recognize the hosts foreign HLA (specifically HLA-D) antigens and react by attempting to reject the host. The severe form of the GVHD reaction affects virtually every tissue and is usually lethal. The occurrence of GVHD disease represents the major obstacle to successful bone marrow transplantation among donors and recipients that are not HLA matched. The major focus of this contract is to develop ways to eliminate GVHD causing cells from bone marrow,

and to be able to treat individuals with GVHD using methods to regulate and eliminate the GVHD causing cells without destroying the capacity of the immune system to protect the individual. The availability of such immunoregulatory tools would make bone marrow transplantation a clinically useful form of therapy in the treatment of irradiation casualties. To develop these immunoregulatory tools, a basic understanding of the human immune response is essential.

The immune system has evolved as a complex network of interacting cells and soluble factors with the capacity to respond rapidly and specifically to virtually all foreign macromolecules (antigens). It achieves this by the very nature of its composition, a multiplicity of sets of cells (clones) each specific for an individual antigenic determinant. Superimposed on the uniqueness of specificity is the ability of different clones to display different effector functions. It is this that bestows such versatility on the repertoire of immune responses. Antigen, although responsible for the induction of clonal expansion is not the only component involved in immune regulation. Clearly, if totally dependent on regulation by antigen, situations could arise where the entire immune system was committed. To prevent this occurring suppressor pathways subtlely interact with inducer populations to provide feedback inhibition. So not only does the suppressor pathway act on the inducer population, but in turn requires that population for its activation. Thus circuits of helper, amplifier and suppressor cells have evolved that regulate both specific antibody production and cytotoxicity. Furthermore, the antigenic specific of the circuits is often the same as that

of the effector cell or molecule. Indeed, antigen bridging between effector and regulating cells is one of the major ways by which the regulating cell selects the appropriate effector cell target. There is now evidence to suggest that regulatory cells may mediate their effects by interacting with antigenic determinants (idiotypes) found on the variable regions of antigen-binding receptors. The regulation of the immune response through idiotype-anti-idiotype interactions would take place independently of antigen and, thus, maintain the immune system in steady state after antigenic stimulation has been withdrawn. This network of idiotype-anti-idiotype interactions would allow diverse members of the immune system to be linked so that the activation of one clone would have far reaching efforts elsewhere within the immunological network. This is the basic principal of the "Network Theory" postulated by Jerne. It has been the aim of this project to prepare anti-idiotype antibodies that mimic these naturally occurring regulatory pathways and to be able to manipulate the immune system. Such reagents would allow the elimination of clones, for example, reactive to a particular transplantation antigen thus allowing transplantation to occur without tissue rejection or graft versus host disease. The blocking effect would be specific for that clone of cells and thus other clones essential for protection against infectious agents would remain unaltered.

In addition to the specificity displayed by the regulatory cells of the immune system it has now become very clear that gene products of the major histocompatibility complex (MHC) are of fundamental importance in cell interactions. Regulatory T cells are not triggered by antigen in its native

form, but require a complex of antigen and MHC antigen, such as Ia, (DR in humans) in order to trigger their receptor. This complex recognition pattern dictates the genetic restriction seen in many cell interactions and elaborates the specificity of the regulation of the immune response. So clearly investigation of the regulation of the immune response must also involve study of those determinants of the MHC also involved.

D. RESEARCH DESIGN AND PLAN

The development of this research contract has been to facilitate the study of the regulatory mechanisms of the immune response. The overall research plan has been divided into a number of areas:

(1) To develop reproducible and reliable *in vitro* techniques for the study of immune responses and to investigate methods for the manipulation of these responses.

(2) Prepare reagents (heterologous and monoclonal antibodies) that recognize cell surface structures (histocompatibility and differentiation antigens) and interaction molecules (receptors and soluble factors).

(3) Analyze the potential enhancing and inhibitory effects of these reagents on immunoregulatory circuits. Furthermore, to attempt to characterize cell interaction molecules using these monoclonal reagents.

(4) Develop antisera that recognize not just the interaction molecules per se but the variable regions associated with these molecules that defines their uniqueness (anti-idiotypic antibodies).

(5) Evaluate the biological activity of anti-idiotypic antisera on cell and factor mediated immune interactions.

The research initiated primarily using rodent models was planned to encompass the regulation of primate and human physiology.

E. RESULTS

(1) Development of *in vitro* technology

The advantages of studying *in vitro* as opposed to *in vivo* immune responses are clear. By their very nature of being *in vitro*, each of the parameters can be precisely controlled and the cell types involved clearly defined. A major focus of our initial work was oriented toward reproducing, in human the *in vitro* systems upon which many of the initial observations were made in animal models. However, the primary sensitization of human lymphocytes *in vitro*, using pariculate and soluble antigens, has not been without its difficulties. Why this is so is unclear, although one of the problems is the reduced viability of human lymphocytes in culture, and our ability to utilize only peripheral blood lymphocytes.

We have been able to develop successful protocols for the in vitro stimulation of human peripheral blood lymphocytes to a variety of antigens such as influenza virus, tetanus toxoid, the synthetic polypeptides (TGAL and GAT) as well as the protein keyhole limpet hemocyanin (KLH). The specificity of these responses was determined by the incorporation of ^{3}H thymidine following stimulation with the appropriate antigens. A preliminary report on this has been published (Lamb et al 1981. Attachment 1). In addition, we currently have a human microculture system for inducing the in vitro synthesis of influenza virus specific antibody, which can be detected and quantitated by means of an ELISA (enzyme-linked immunosorbent assay).

As described in detail in Technical reports 2 and 3, the modified Marbrook-Diener tissue culture system has been used for the production and assay of human helper factors. We have now adapted this system for the production and assay of suppressor factor molecules (Kontiainen et al 1981, Attachment 2). The analysis of these molecules is discussed in Results Section 4.

The development of these in vitro assay systems has been essential to provide models of normal human immune function upon which to determine the activity of anti-receptor and anti-interaction molecule reagents.

(2) Preparation of biological reagents against cell surface structures and interaction molecules

a) Development of monoclonal antibodies

As described in technical report 3 it was our intention to develop the capacity to produce monoclonal antibodies using somatic cell hybridization

techniques. Having screened and isolated those antibodies with functional activity, they were to be injected into the appropriate recipients in an attempt to generate anti-idiotype antibodies. Indeed we now have stable hybridomas against the following antigens:

1) Sheep red blood cells
2) TGAL
3) KLH

The cell lines have been expanded to large number and frozen at -170°C in liquid nitrogen. In addition to these clones a variety of other hybridomas have been obtained that recognize surface determinants on murine or human lymphocytes.

1) anti-Ly1 hybrids
2) anti-Ly2 hybrids
3) anti-Thy-1 hybrids
4) anti-HLA-A, B and C common
5) anti-human $_2$ microglobulin

The anti-Ly1 and Thy-1 reagents are invaluable as phenotypic markers for the analysis of lymphocyte subpopulations in the murine system.

We are currently attempting to develop monoclonal antibodies that recognize polymorphic HLA-DR region determinants, since these molecules behave as restriction elements and appear to be intimately involved in cell interaction.

In addition, purified hybridoma ascites for the murine cell surface antigens Ia^k and Ia^s, rat T cell and Ia antigens have been secured.

for the analysis of cell interactions. Over the past year we have accumulated ten monoclonal antibodies against the human DR region molecules. These are currently undergoing testing in our systems.

b) Functional studies to test the activity of reagents that recognize cell interaction molecules

i) Mixed lymphocyte response: An assay system for the mixed lymphocyte response of rat peripheral blood lymphocytes or spleen cells as responder cells, and spleen cells as stimulator cells has been developed (Bash et al 1980, Attachment 3). Such an assay allows the influence of anti-cell interaction molecule reagents on subsets from different lymphatic organs to be investigated. The differential effects of two antisera that recognize rat T cells were assessed in the MLR. One anti-T cell antisera (W3/25) inhibited the proliferation of the responder cells but the other W3/13 did not, suggesting they recognize discrete T cell subsets one of which is important in MLRs (ie. specific for alloantigen recognition). This is supported by the observation that neither antisera inhibited the response to the T cell mitogen PHA. (Bash et al. 1980, Attachment 3). Furthermore, an anti-Ia antisera also inhibited the MLR of peripheral blood and spleen lymphocytes implying either a subset of Ia bearing T cells are present both peripherally in the blood and centrally in the spleen, or that the inhibition is at the level of the macrophage.

ii) Antigen-specific proliferative responses: Having developed an *in vitro* system for the proliferative response of human lymphocytes to a

variety of antigens, it is was used to determine the effects of heterologous and monoclonal antibodies that recognize DR framework determinants. Both the rabbit anti-DR and monoclonal anti-DR antibodies were able to inhibit tetanus toxoid and influenza virus specific proliferation (Lamb et al. 1981, Attachment 4). Other antisera directed against T cells and $_2$ microglobulin have been added to these antigen specific proliferative responses. This work, in an expanded version, is currently being written up for publication.

iii) Antigen specific helper and suppressor assay systems: Assay systems have been established *in vitro* in Marbrook chambers for the analysis of helper and suppressor molecules. The effect of antisera with the potential to modulate cell interactions can be assessed in two ways. Firstly, they can be added directly to the culture system together with the helper or suppressor molecules, and secondly they can be coupled to sepharose beads and the resulting solid phase immunoadsorbents used to bind the functional activity of the factors. Results on the reactions of various antisera and helper and suppressor factor are discussed in Results Section 4).

(3) Development of anti-idiotypic antibodies specific for cell interaction molecules.

One of the major problems that has hindered the development of antisera specific for T cell receptors has been the inability to obtain purified receptor either as an isolated molecule or in situ on the T cell itself. However, with the recent advent of T cell cloning it is now possible

to obtain expanded populations of homogeneous T cells. We have successfully cloned influenza virus specific human T lymphocytes (Lamb et al. 1981, Attachment 1). These clones are now being subjected to intensive analysis of their specificity and functional properties (ie. help, suppression, proliferation and cytotoxicity). Expanded populations of the individual cell types will be used to immunize mice and develop anti-receptor antibodies, which in turn will be used to generate anti-idiotypic antibodies. In a similar fashion the anti-DR antibodies and cloned primed lymphocyte typing cells described in results (Section 2) will be used to develop anti-idiotypic reagents. Furthermore, these reagents when developed will be assayed on antigen-specific clonal immune responses rather than the response of unfractionated peripheral blood lymphocytes and this revealed a much clearer analysis of the cell types involved.

(4) Characterization of cell interaction molecules.

Helper and suppressor molecule production and assay systems have been established *in vitro* in Marbrook chambers for a variety of antigens, KLH, GAT, TGAL and antigens from pathogenic bacteria such as *Streptococcus mutans*. Furthermore, we now have these systems functioning for the analysis of helper and suppressor molecules derived from mouse, monkey and human lymphocytes. Much on the nature of the helper factor molecules have been described in Technical reports 2 and 3. However, we have additional information on specific helper factor, namely that it contains determinants cross reactive with 2 microglobulin (Lamb et al. 1981, Attachment 5). In addition, to analyze the helper factors that induce B cells to synthesize specific antibody, we have

partially characterized molecules that suppress antibody production by their action on helper cells (Kontiainen et al. 1981, Attachment 2). These studies reveal that suppressor factor:

a) Suppresses specific antibody production at the helper T cell level.

b) Antigen-specific in its activity

c) Contains Ia like encoded determinants of the HLA-DR region

d) Contains a "constant" region discrete from that present in helper factor as defined by rabbit anti-helper and anti-suppressor factor antisera.

F. CONCLUSION FROM COMPLETED WORK

The research completed to date has provided us with valuable information on the regulatory mechanisms of the human immune responses. The most significant advances have been in the analysis of human cellular immune responses. The development of *in vitro* assay systems and the cloning of specific human T lymphocytes have provided us with two powerful tools for the development and functional analysis of anti-receptor (anti-idiotype) antibodies. Furthermore, our data confirms and extends the importance of D region encoded products of the MHC in the regulation of human immune responses at the level of the T cell-macrophage interaction and in the production of specific antibody. The techniques previously performed for the analysis of the nature of human antigen-specific helper factors has been extended to encompass suppressor factor molecules; and the physicochemical properties of these

molecules has now been determined. Thus, the data generated on this research contract over the last nine months has significantly advanced our understanding of the nature of the regulation of human immune physiology. Only now, that our technology (human T cell cloning and in vitro assay systems) has evolved can we develop and analyze with any confidence biological reagents specific for cell interaction molecules.

G. PROPOSAL FOR CONTINUATION

In view of the success in cloning human T lymphocytes and developing in vitro human immune responses, together with the critical data on the manipulation of these responses has encouraged us to continue this line or research. It is now proposed using these advances to further develop reagents as outlined below, that recognize lymphocyte surface structures and interaction molecules, and are thus capable of effecting clonal elimination.

Work Plan:

1) Studies on the development of anti-idiotype antibodies to monoclonal antibodies. During the next contract year monoclonal reagents will be sought that are capable of blocking specific primed lymphocyte typing (PLT). The reagents will be used in the preparation of anti-idiotype antibodies. Depending upon the success of this approach, these anti-idiotypic reagents will be tested for activity against normal immune cells, and for their effect in

moderating normal immune reactivity. The site of blocking of the PLT reaction will also be investigated. Similar studies will be performed, as a non MHC antigen control with influenza clones and monoclonal antibodies.

2) Studies on the development of anti-receptor antibodies to cloned PLT cells. Rats will be tolerized in utero to human cells, and after birth immunized with cloned PLT cell lines. After an appropriate immunization schedule the spleens will be removed and fused with a rat hybridoma line. The resultant monoclonal antibodies will be screened for activity against the receptors on the cloned PLT cells by their ability to block its proliferative activity. Similar studies will be performed with influenza clones as a non MHC antigen control.

3) Studies on normal immune responses. The normal human immune response to a variety of antigens, including influenza, tetanus and other related agents will continue to be evaluated. The assays developed to test for T cell proliferation and antibody production will be used to analyze the functional activity of monoclonal antibodies. The cell populations involved in these interactions will also be evaluated using the Fluorescence Activated Cell Sorter. Furthermore, antigen-specific cloned human T cells will be analyzed for their ability to synthesize cell interaction molecules and used to generate anti-receptor antibodies.

PUBLICATIONS

1) Lamb, J.R., D.D. Eckels and J.N. Woody (1981). Antigen-specific human T lymphocyte clones: 1. Isolation, growth and charcterization of influenza virus-specific clones. J. Exp. Med. (Submitted).

2) Lamb, J.R., E.D. Zanders, A.R. Sanderson, P.J. Ward, M. Feldmann, S. Kontiainen, T. Lehner and J.N. Woody (1981). Antigen specific helper factor reacts with antibodies to human β microglobulin. Journal of Immunology (In Press).

3) Kontianen, S., Woody, J., and Feldmann, M (1981). Human suppressor factors. Clinical Exp. Immunol.

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1) Bash, J.A., S. Shapiro, L.E. Adams and J.N. Woody. (1980). Inhibition of mixed lymphocyte response by monoclonal antibodies specific for rat lymphocyte subsets. Third International Workshop on Alloantigenic Systems in the Rat. Philadelphia, PA. June 1980, Abst. 22.

2) Lamb, J.R., M. Brunswick, E. Ketterer and J.N. Woody (1981). Inhibition of human proliferative responses to antigens and mitogens by irradation and anti DR antibodies. Federation Proceedings 40, 1033.

ATTACHMENT NO. 1

ANTIGEN-SPECIFIC HUMAN T-LYMPHOCYTE CLONES: 1. The isolation, growth and characterization of influenza virus-specific clones

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* These studies were supported by funds received from the Office of Naval Research Contract N000-14-77-C-0748 Research Task No. NR 207-102 and Contract N000-14-77-C-0747 Research Task No. NR 207-101. The opinions expressed herein are those of the authors and are not to be construed as those of the Department of the Navy or the Department of Defense.

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Running Title: Influenza-specific human T-lymphocyte clones.

Abbreviations used in this paper: AET-SRBC, S-2-aminoethylisothiouronium bromide treated SRBC; APC, antigen presenting cell; E, erythrocyte; E+, mononuclear cells which form rosettes with AET-SRBC; E-, non AET-SRBC rosette forming cells; FCS, fetal calf serum; GAT, L-Glutamic acid^{60}: L-Alanine^{30}: L-Tyrosine^{10}; ^{3}HTdR, tritiated methylthymidine; TGAL, (L-Tyrosine: L-Glutamic acid) Poly DL-Alanine:Poly-L-Tryosine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid; HLA, major histocompatibility complex of man; KLH, keyhole limpet hemocyanin; PBL, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PPD, purified protein derivative; PWM, pokeweed mitogen; RI, reactivity index; SRBC, sheep red blood cells; TCGF, T-cell growth factor; TLC, T-lymphocyte clone; T. Tox., tetanus toxoid; HA, hemagglutinin; NA, neuraminidase; MP, matrix protein.

INTRODUCTION

The analysis at the molecular level of T-cell interactions in the regulation of immune function requires expanded populations of monoclonal T-lymphocytes. A number of approaches have been utilized to obtain enriched populations, for example, antisera reactive with cell-surface determinants have defined the characteristics of some T-lymphocyte subsets both in mouse (1,2) and in man (3-5). Using such antisera, certain T-cell types can be enriched by either positive or negative selection (6-8), although these techniques fail to isolate monoclonal populations. In another approach, somatic cell hybridizations between normal murine T-cells and thymomas resulting in T-cell hybrids with specific suppressor (9,10) or helper (11) activity have been reported. However, such techniques are not sufficiently developed to allow the monoclonal expansion of all T-cell subsets, and are currently not available for analyzing human T-lymphocyte function. Recently, the recognition that supernatants from mitogen stimulated lymphocyte cultures (T-cell growth factor, TCGF) can maintain the growth of T-lymphocytes in vitro (12,13), has allowed the expansion and maintenance of functional antigen-specific T-cell subpopulations in long-term culture (14-17). Consequently, in the mouse it has been possible to prepare antigen-reactive clones of T-cells that recognize specific alloantigenic determinants (18,19), particulate antigens such as sheep erythrocytes (20) or soluble protein antigens (21,22).

In the human, the majority of early studies utilized proliferation of PBL populations to antigens such as purified protein derivative (PPD) to analyze cell interactions (23,24). More recently, long-term cultures of human T-lymphocytes which are dependent upon TCGF for continued growth and which are specific for soluble antigens such as PPD and tetanus toxoid (T. Tox.) have been reported by Kurnick et al (25,26). It has also been demonstrated that human T-cell lines specific for soluble antigens (27) or alloantigens (28) can

be cloned. In the studies reported here, we describe the system for the generation and characterization of T-lymphocyte clones (TLCs) which have antigen specificity for influenza viral proteins.

MATERIALS AND METHODS

Cells. Mononuclear cells from peripheral blood (PBL) were obtained from whole blood diluted with an equal volume of RPMI 1640 (Grand Island Biologicals Company, Grand Island, NY) and centrifuged over Ficoll-Hypaque (Sigma Chemical Company, St. Louis, MO and Winthrop Laboratories, New York, NY) at 400g for 30 minutes. After washing, the cells were resuspended to 10^7/ml in RPMI 1640 medium supplemented with 10% screened, pooled human A^+ serum, 2mM L-glutamine, 25mM HEPES buffer, 50μg/ml gentamycin, 25 IU/ml sodium heparin, 1mM sodium pyruvate and 7.5% v/v dimethylsulfoxide at 4°C. The cells were frozen at -1°C/min for 20 minutes using a rate-controlled freezer (Cryson, Associated Biomedic Systems, Buffalo, NY), then at -50°C/min down to -80°C. Following this procedure, the cells were transferred to the vapor phase of a liquid nitrogen freezer (MVE Cryogenics, New Prague, MN) and stored at -180°C until required.

Antigens. Attenuated influenza A (A/Texas/1-77/x-[illegible]) and B (B/Singapore/222/79) viruses and isolated hemagglutinin (A/Bangkok/79) were kindly prepared by M. Phelan and W.E. Barthlow, Division of Virology, Bureau of Biologics, NIH, Bethesda, MD. Isolated neuraminidase (Papau New Guinea/1/75) and matrix protein (A/Bangkok/79) were generously provided by Dr. R. G. Webster, St. Jude Children's Research Hospital, Memphis, TN. The polymers L-Glutamic $acid^{60}$:L-$Alanine^{30}$:L-$Tyrosine^{10}$ (GAT) and (L-Tyrosine: L-Glutamic acid) Poly DL-Alanine: Poly-L-Tyrosine (TGAL; Lot MC8) were purchased from Miles Laboratories, Inc., Miles Research Division, Elkhart IN. Keyhole limpet hemocyanin (KLH) was the generous gift of Dr. M. Rittenberg, Portland, OR. Tetanus toxoid (T. Tox.) was purchased from Massachusetts Biological Laboratories, Boston, MA.

Antigen Activation of PBLs. PBLs were diluted to 10^6 cells/ml in RPMI 1640 (GIBCO) containing 10% screened, pooled human A^+ serum, 2mM L-glutamine, 25mM HEPES buffer, 50μg/ml gentamycin, 25 IU/ml Na-heparin and 1mM Na-pyruvate. Cells were suspended with an equal volume of medium containing five hemagglutinating (HA) units/ml of influenza A virus. The concentration of virus required to produce optimal stimulation of PBLs, assayed by ^{3}HTdR-incorporation, varied for different virus preparations, but was always in the range of 2-5 HA/ml. Hemagglutinin (HA) was used at 0.1μg/ml, neuraminidase (NA) at $5x10^{-4}$ vol % and matrix protein (MP) at 0.1μg/ml. These concentrations were previously determined to produce optimal stimulation. After mixing, 0.2ml aliquots of the cell suspensions were plated into 96 well, U-bottom tissue culture trays (Linbro Scientific Company, Hamden, CT) and incubated for six days at 37°C in a 5% CO_2/air mixture.

T-Cell Growth Factor (TCGF). PBLs from screened donors were cultured at $1x10^6$/ml in RPMI 1640 supplemented with 0.1% purified phytohemagglutinin-P (PHA-P; Difco Laboratories, Detroit, MI) and 1% autologous plasma (29). After 48 hours, supernatants were harvested, passed through 0.22μm filters and assayed for their ability to support the growth of a TCGF dependent cell line as assayed by tritiated thymidine incorporation. Acceptable lots of TCGF were stored at 4°C or diluted as required to 20% v/v in 10% AB plasma and supplemented RPMI 1640.

Cloning of Influenza Specific Lymphocytes. After six days culture in the presence of influenza virus, cells were harvested, resuspended over 35-40% Percoll (Pharmacia, Uppsala, Sweden) in 12x75mm sterile test tubes (Falcon Division, Becton Dickinson and Company, Cockeysville, MD) and centrifuged for

20 minutes at 200g. Cells at the interface were enriched 2-5 fold for lymphoblasts and comprised 50-70% of the cells counted. Blast-enriched suspensions were diluted to 33 1/3 cells/ml of medium, containing 20% TCGF and plated in 10μl aliquots in sterile 60-well Microtest II trays (Falcon). Ten thousand autologous cells were γ-irradiated (2500 rads, ^{137}Cs), combined with optimal concentrations of influenza virus and added to wells containing blasts in 10μl aliquots. Cultures were incubated for seven days in humidified chambers at 37°C in 5% CO_2/air after which growing wells were transferred to fresh medium (0.2ml) containing 20% TCGF, irradiated autologous feeders ($5x10^5$ml) and influenza virus in 96-well flat-bottom trays. Following seven additional days of cultures, the clones were transferred to 24 well trays (Linbro Scientific Company, Hamden, CT) containing the appropriate concentrations of TCGF, autologous feeders and influenza in a total volume of 2ml. Cultures received fresh TCGF every 3-4 days alternating with pooled irradiated feeders ($5x10^5$/ml) without virus and were thus maintained throughout the course of the experiments. Clones were allowed to grow 6-8 days following addition of feeder cells prior to testing in proliferative assays.

Preliminary Screening for Influenza-Responsive Clones. Individual clones from 24-well trays were resuspended with a Pasteur pipette and diluted 1:20 in supplemented RPMI 1640. Aliquots of 0.1ml were added to each well of a 96-well U-bottom tray, each of which had received 0.1ml of autologous γ-irradiated PBL ($2.5x10^5$/ml) and influenza A virus (5 HA units/ml) in 10% A+ serum and supplemented medium. Controls consisted of TLC and influenza without autologous PBLs, TLC and PBL without influenza, TLC alone and TLC with 10% TCGF. Cultures were incubated for 72 hours followed by an 8 hour pulse with 1μCi of tritiated methylthymidine (^{3}HTdR, New England Nuclear, Boston, MA).

Triplicate samples were harvested onto glass-fiber filters and radiolabel incorporation was quantitated by liquid scintillation spectroscopy.

Proliferation Assays. Five thousand TLC cells in 10% A+ serum and supplemented medium were added to 96-well, U-bottom trays in 0.1ml aliquots. Fractionated or unfractionated autologous PBLs, as a source of antigen presenting cells (APC), were suspended in medium containing 10% A+ serum and optimum concentrations of antigen and dispensed in 0.1ml aliquots to wells containing TLC cells. Cultures were incubated for 12-144 hours, pulsed for 8-16 hours with 1.0μCi of ^{3}HTdR and harvested onto glass-fiber filters. Proliferation, as correlated with ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (cpm) $\pm$ standard error of the mean (SEM) for triplicate cultures.

Fractionation of Lymphocyte Populations by Sheep Erythrocyte Rosetting. Erythrocyte (E) rosettes were formed with sheep red blood cells (SRBC) treated with AET (S-2-aminoethylisothiouronium bromide hydrobromide, Calbiochem, San Diego CA) as previously described (30). One volume of packed SRBC was incubated with five volumes of AET (40.2mg/ml in distilled H_2O, pH 9.0) for 20 min at 37°C. One volume of PBLs at 10^7 cells/ml was mixed with two volumes of 2% AET-SRBC and 0.5 volumes of fetal calf serum (FCS). The suspension was centrifuged at 250g for 10 minutes, and placed on ice for 60 min. After resuspension of the pellets by gentle rotation, the separation of E rosette forming (E+) from nonrosetting (E-) lymphocytes was achieved by centrifugation (1500g, 15 min) over Percoll (p=1.080 g/ml; Pharmacia). The E^- cells were recovered from the interface and the E^+ cells from the pellet by lysis of SRBCs with Gey's hemolytic solution (31). The E- cell population

contained less than 1% E+ cells. In proliferation assays, cultures were reconstituted with $5x10^3$ E-, $20x10^3$ E+, or $25x10^3$ E+ and E- cells. Comparable cultures were reconstituted with $25x10^3$ irradiated PBL as a source of antigen presenting cells (APC).

Characterization of TLCs by FACS Analysis. Clone cells were pelleted by centfifugation at 300g for 10 min at 4°C and then resuspended to 10^7 cells/ml in ice-cold supplemented medium containing 10% FCS plus 1mg/ml Na azide. To 100µl aliquots of cells, 5µl of monoclonal antibody was added and incubated for 30 min with agitation every 10 min. Following two washes in cold medium, 10µl of counterstaining reagent (either FITC-$F(ab')_2$-sheep anti-mouse-IgG, Cappel Laboratories, Cochranville, PA or FITC-conjugated avidin, see below) was added, incubated for 30 minutes on ice with agitation and washed twice. Cells were resuspended in 2ml of medium and analyzed for fluorescence on an Ortho Fluorescence Activated Cell Sorter, Model 50H (Ortho Diagnostics, Boston, MA). The murine monoclonal antibodies and reagents used in these characterizations were: OKT3,4,6,8 (Ortho); biotin-conjugated Leu 2A,3A,αDR and FITC-avidin and FITC-Leu-1 (Becton Dickinson, Sunnyvale, CA); αHLA-framework (Bethesda Research Laboratories, Bethesda, MD); FITC-conjugated, pooled, goat anti-human-Ig ($F(Ab')_2$; Kallestad Laboratories, Inc., Chaska, MN).

RESULTS

Cloning Efficiency. Following initial stimulation with virus, lymphoblasts were placed in Terasaki plates where 12% of the wells contained growing cells after seven days. Since the blast-enriched cells were plated at one cell every third well, approximately 35% of the total number of seeded cells proliferated. Furthermore, upon subsequent transfer to 96-well trays, 100% of the clones were still growing after seven additional days in culture.

Preliminary screening for influenza responsive clones. A total of 96 individual TLCs were cultured in the absence of TCGF with autologous irradiated PBLs as a source of antigen presenting cells (APC) and their responsiveness to influenza A virus was determined by ^{3}HTdR incorporation. In order to concentrate our efforts on only positive clones, TLCs were grouped into four categories as assessed by the magnitude (cpm) of the proliferation (Table 1). The distribution of clones within these categories was as follows: 27 in group I (1-500 cpm); 27 in group II (500-3000 cpm); 27 in group III (3000-10,000 cpm); and, 15 in group IV (>10,000 cpm). Since the number of cloned T-cells added to this proliferation assay was not determined because of the large number of clones being screened, equal volumes were also cultured with TCGF to give an indication of both the cell number and the proliferative potential of individual clones. The ratio of the response of each clone stimulated with influenza in the presence of autologous irradiated PBL to that of the same clone stimulated with TCGF alone was determined and referred to as the reactivity index (RI):

$$\text{Reactivity index} = \frac{\text{TLC+ influenza + PBL}}{\text{TLC + TCGF}}$$

An index of one or greater would indicate that antigen specific proliferation was occurring. The reactivity index of each TLC was determined and placed in

one of three groups, A(0-0.5), B(0.5-1.0) or C (1.0), and correlated with the location of that clone in Groups I-IV (Table 1). Of the 27 clones in Group I, 25 were also located in RI group A, 1 in Group B and 1 in Group C. In Group II, however, 26 of the 27 clones were in Group A with the remaining clone in Group B. Whereas in Group III the majority of the clones located in RI Group C, with 4 and 3 clones in Group A and B respectively. All of the clones in Group IV were also in RI Group C. By virtue of their location in Groups I-IV and RI Group A, B or C, the population of clones was divided essentially into positive and negative clones with only a few intermediates. Positive clones were considered to be those in Groups III or IV and in RI Group C. Negative clones, however, were considered to be those in Groups I or II, and also in RI Group A.

The proliferative response of a selected group of clones is shown in Table 2. By fulfillment of the requirements outlined above, TLCs 18, 50, 76 and 77 were considered negative, while the remainder (TLCs 6, 24, 37, 53, 71, 69, 72 and 88) were considered positive. From this panel of clones, seven positive clones (TLCs 6, 24, 26, 37, 53, 71 and 72) and one negative (TLC 50) clone were analyzed in the following experiments.

Kinetics of TLC proliferation to influenza A virus. The clones selected from the preliminary screening were cultured with antigen presenting cells and influenza A for varying periods of time. The proliferative response of TLCs was determined at 12, 24, 36, 48, 60, 72, 96 and 144 hours after the initiation of culture (Figure 1). Of the positive clones selected, all reached maximum ^{3}H-thymidine incorporation by 96 hours. Proliferation at 144 hours was not significantly above the background response. TLCs 6, 26, 37, 71 and 72 responded optimally between 72 and 96 hours, although the magnitude of the response varied for each clone, ranging from 15x10^3 cpm (TLC 72) to 57x10^3 cpm (TLC 26). Maximum incorporation of ^{3}H-thymidine for clones 24 and 53

occurred earlier at 36 and 60 hours, respectively (Figure 1). Throughout the time course of the experiment, the negative clone (TLC 50) did not proliferate significantly when presented with influenza A.

At 12 hours, all TLCs proliferated more vigorously in response to TCGF than to specific antigens. However, at 24 hours the response to specific antigen was equal to or greater than that to TCGF alone, and by 72 hours was 5-fold greater than the non-specific response to growth factor. One exception was TLC 50 which at 144 hours responded much more vigorously to TCGF than to specific antigen, suggesting that the clone was truly negative and that its failure to respond did not reflect cell death. Proliferation of TLCs in the absence of specific antigen and TCGF was also monitored at each time point, and for the majority of the clones this remained below 100 cpm. TLC 26, however, proliferated vigorously in the absence of stimulation for the first 48 hours, and even at 72 hours gave a count of 640 cpm. This was also observed with clone 24, but to a lesser degree, with unstimulated proliferation diminishing by 48 hours.

The maximum ^{3}H-thymidine incorporation varied considerably for individual TLCs, considering that an equal number of cells was used in each experiment. However, of seven positive clones reported in the present study, broad grouping into two categories was possible: those with peak proliferation in excess of 40×10^3 cpm (TLCs 6 and 26) and those with $15\text{-}20 \times 10^3$ cpm maximum proliferation (TLCs 24, 53, 71 and 72). There was one intermediate responder (TLC 37) with a maximum response of 30×10^3 cpm at 72 hours.

Antigen specificity of T-lymphocyte clones. The antigenic specificity of individual TLCs was assayed by culturing clones with autologous PBLs and various antigens (Table III). Although all positive clones (TLCs 6, 24, 26, 37, 53, 71) proliferated vigorously to influenza A virus, each TLC was also

restimulated by at least one apparently unrelated antigen. Such ancillary rections were generally less than 10% of the response to influenza A, however, they were significantly above background controls. To explain these data, three possibilities seemed evident: a) antigen-specific precursors in the PBL presenting population were reacting to these antigens and producing "back stimulation" via release of growth factors; b) although the TLCs were clones, they were detecting cross-reactive determinants; or, c) the TLCs were not actually clones, but contained cells with other reaction patterns. We hypothesized that "back stimulation" seemed the most plausible alternative and reasoned that removal of T-cells from the presenting cell population should eliminate "non-specific" responsiveness.

When peripheral blood T-cells were removed from PBL by rosetting with AET-treated SRBC, TLCs presented with antigen by the remaining E^- APC showed little or no cross-reactivity; because of the large quantity of data, typical results are presented only for TLC 53 (Table IV). Summary results of antigen presentation by E^- APC are presented in Table V. Without exception, TLCs were not reactive to unrelated antigens when presented in the absence of T-cells.

In order to define more precisely the antigen specificity of TLCs, clones were restimulated with viral subcomponents presented by E^- cells (Table VI); data from four clones (TLCs 6, 26, 37 and 71) are shown. TLCs 6, 26, 37 and 71 all proliferated when presented with complete influenza A virus (9,843 $\pm$ 504 cpm, 7,182 $\pm$ 1,728 cpm, 18,191 $\pm$ 479 cpm and 8,570 $\pm$ 2,018 cpm, respectively) but not influenza B virus. Of eight tested, no TLC responded to hemagglutinin, TLCs 6 and 37 responded to matrix protein (4,132 $\pm$ 751 and 9,752 $\pm$ 1,677 cpm, respectively) and TLCs 26 and 71 were stimulated by neuraminidase (3,680 $\pm$ 344 and 5,291 $\pm$ 1244 cpm, respectively). The negative control (TLC 50) responded only to TCGF (data not shown).

Immunofluorescence Characterization of TLC Surface Markers. TLCs 6 and 37, the most reactive clones, were characterized using a panel of monoclonal anti-T-cell antibodies and the fluorescence activated cell sorter (Table VII). Both clones were positive with OKT3 (pan-T-cell) as well as for HLA and DR antigens. Approximately 50-80% of the cells in each TLC were reactive with the OKT4 and Leu 3A reagents which allegedly detect an inducer-helper type marker. Cells from neither clone were stained by OKT6 (thymocytes), OKT8 or Leu 2A (suppressor-cytotoxic cells) or anti-sIg (B-cells). Interestingly, Leu 1, a pan-T-reagent which is similar to OKT 3, but which detects a different cell surface molecule, was found on TLC 6, but not on TLC 37. Each TLC formed more than 95% E-rosettes.

DISCUSSION

The *in vitro* generation of discrete subsets of human T-cells with different functions such as help, suppression, cytotoxicity and proliferation has been reported for a variety of antigens (32-35). Furthermore, it is known that long-term cultures of human T-cells with specificity for allogeneic (28,29,36) or soluble antigens (26,27) can be maintained using TCGF. In the present study we report the generation, maintenance and characterization of cloned human T-cells that proliferate specifically in response to influenza A virus.

The unfractionated PBL from normal donors were screened for *in vitro* proliferative responses to influenza A virus. From a selected high responder, unfractionated PBL were cultured with a single pulse of influenza A virus to induce blast transformation of T-cells. These cells were subsequently cloned by limiting dilution thus making it possible to select for antigen specificity prior to clonal expansion. This is similar to the approach used by Kurnick et al. (25,26), for the induction of human blast cells, which were subsequently cloned (27). Murine proliferating T-cell clones, selected for antigen specificity prior to clonal expansion in TCGF have also been reported (22). However, contrary to our findings, the proliferative assay in the murine system required the presence of TCGF. Other techniques have centered on enriching for specific cells by repeated antigenic stimulation and culture in the presence of TCGF (15-17,39). A potential problem with this approach is that the cells that grow well *in vitro* in response to TCGF are not necessarily those cells that are antigen specific.

In the studies reported here, the frequency of growing clones following plating at one cell every three wells, was approximately 35%. Different plating efficiencies have been reported by different investigators in a variety of systems (27,28,36). We feel that such differences are reflected in the nature of the antigens used, the presence of both specific and non-specific recruitable T-cells, quantitative or qualitative differences in the T-cell subsets found in peripheral blood as compared to lymphatic organs, or merely variations in technical procedures. Other problems arise from the question of whether TLCs are actually clones. By Poisson probabilities, 99% of the wells should have contained one or fewer cells[b]. This, in conjunction with the fact that only a subset (35%) of those cells originally seeded produced viable lines, makes it statistically probable that most TLCs are actually clones. In this regard, subcloning of TLCs may resolve concerns regarding the clonal nature of TLCs, but in our hands at this time, this has not been technically feasible.

The preliminary screening of the influenza virus TLCs revealed clear patterns of responsiveness as determined by the magnitude of the ^{3}H-thymidine incorporation and by the reactivity index. The latter was used as an indicator of the cloned T-cell number and their viability. On the basis of these two parameters, 36.5% of the clones were considered to be positive for influenza A, 10.4% intermediate and 53.1% negative. From these, seven positive clones and one negative clone were analyzed in detail. The stimulation of the influenza-virus induced clones with a panel of unrelated antigens resulted in significant proliferation of the clone in response to specific antigen. Additionally, at least one other antigen always restimulated when irradiated autologous PBLs were used as the source of APCs. Three alternatives were considered as possible explanations of those observations. First, that the TLC

[b] Eckels, D.D. and R.J. Hartzman. 1981. Does HLA-D Exist? Human Immunology (Submitted).

were, in fact, not monoclonal and that cells with other specificities were present. Second, that the T-cell clones were monoclonal but cross reacted with determinants shared by the other antigens. Third, that within the presenting PBL population, there was an irradiation resistant cell type capable of triggering proliferation of the TLC in the presence of unrelated antigen. Furthermore, it seemed improbable that all the clones analyzed would have specificities that cross reacted with antigens as diverse as influenza B virus, T. Tox, TGAL, GAT and KLH. In an attempt to resolve this problem, different fractions of PBL were assayed for their ability to act as APCs. It was observed that the addition of an irradiated E-rosette negative fraction consisting primarily of B-cells and monocytes, resulted in the proliferation of TLCs when stimulated with influenza A, but not when cultured with unrelated antigen. Thus, the most likely explanation for the apparent cross reactivity of influenza A specific TLCs was that it was due to the presence of an irradiation resistant T-cell population which upon antigen stimulation, released TCGF or other growth promoters which in turn induced nonspecific proliferation by the TLC. This is supported by reports that irradiation resistant T-cells can release TCGF following antigen stimulation (29) and the observation that allospecific TLCs, which are dependent upon TCGF for growth, also proliferated in the presence of unrelated antigens and irradiated PBL (Lamb and Eckels, unpublished observations).

All of the reactive clones analysed in this report were antigen specific in that they responded to influenza virus strain A, the inducing antigen, but not to influenza strain B or other unrelated protein antigens. Furthermore, with the use of purified viral subcomponents, neuraminidase, haemagglutinin and matrix protein, the fine antigenic specificity of these clones was determined. Interestingly, of the clones tested here, none reacted with hemagglutinin. In this regard, it has been reported that mouse T-cells primed with matrix protein can act as helper-cells to induce antibody response to hemagglutinin (37,38)

suggesting associative recognition of viral components. However as yet, there is no direct evidence that the clones specific for matrix protein which are reported here can cooperate with B cells in the production and hemagglutinin-specific antibody. Nevertheless, in view of their association with the helper/inducer T-cell subsets defined by phenotypic analysis (OKT4$^+$, Leu 3A$^+$) and the observation of Kurnick et al.(27) that antigen specific T-cell clones which proliferate strongly are also helper-cells in the production of antibody, suggests a similar mechanism.

In addition to the ability of these TLCs to proliferate in response to influenza A, they are being analyzed for other functions such as help, suppression and cytotoxicity, with the proviso that culture conditions for the production of proliferating T-cells may not necessarily be those for the induction of other subsets with different function. Although it appears that human T-cell clones that proliferate strongly in response to specific antigen also display helper and suppressor activity in polyclonal B-cell responses (27). This is in contrast to mouse antigen specific T-helper clones which clearly have different culture requirements than other functional subsets (39). Finally, human antigen specific T cell clones are a powerful tool and should allow the detailed analysis of many aspects of lymphocyte interactions, previously impossible with conventional cell culture techniques.

SUMMARY

Human peripheral blood lymphocytes were primed in vitro with influenza A virus (A/Texas/1-77/x-49) and subsequently cloned by limiting dilution in TCGF. Although 96 TLCs were originally obtained, eight were characterized in detail. Kinetic experiments revealed that optimal proliferation occurred at approximately 72-96 hours following presentation of specific virus by autologous PBLs. The maximum proliferation varied according to the individual TLC being tested and ranged from $15x10^3$ cpm to more than $60x10^3$ cpm. TLCs were found to respond specifically to influenza A virus, not to influenza B, TGAL, GAT, tetanus toxoid or KLH, and only when antigen was presented by cells unable to form rosettes with AET-treated SRBCs. Presentation of antigen (T. Tox. in particular) by PBLs, often resulted in significant "back stimulation", probably via production of growth factors. In addition, the antigen specificity of these clones for viral subcomponents was directed towards matrix protein and neuraminidase but not hemagglutinin. By FACS analysis, using commercial monoclonal anti-T-cell antibodies, two TLCs were $OKT3^+$ (pan-T-cell), $OKT4^+$ and Leu $3A^+$ (inducer/helper subset), HLA^+, DR^+, $OKT6^-$, $OKT8^-$, Leu $2A^-$ and sIg^-; only one of the clones expressed the pan-T Leu-1 antigen.

Acknowledgements: The authors thank Ms. E. Ketterer for expert technical assistance and Ms. B. Minor for secretarial help. Special thanks are due Dr. R. Hartzman for running the fluorescence activated cell sorter and to Drs. P. Lake and J. Neefe for helpful discussions and critical review of this manuscript.

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Legend to Tables

Table 1.

Unfractionated PBL from a high responder were stimulated with influenza A virus and cloned by limiting dilution. 96 colonies were picked and expanded in liquid culture. A 1:200 dilution of T-cells from each colony were stimulated with 5HA units/ml of influenza A in a 72 hours ^{3}H-thymidine incorporation assay in the presence of $25x10^3$ irradiated (2500 rads) autologous PBL

a. Reactivity index = $\frac{\text{TLC + influenza A + PBL}}{\text{TLC + TCGF}}$

Table II

Unfractionated PBL from a high responder were stimulated with influenza A virus and cloned by limiting dilution. 96 colonies were picked and expanded in liquid culture. $5x10^3$ T-cells from 13 of the colonies were stimulated with 5HA units/ml of influenza A in a 72 hour ^{3}H-Thymidine incorporation assay in the presence of $25x10^3$ irradiated (2500 Rads) autologous PBL. The response of high and low responder clones is shown. Controls of irradiated autologous PBL with flu, PWM, and PHA are shown. The values in the parentheses are standard errors of the mean of triplicate cultures.

Table III

Unfractionated PBL from a high responder were stimulated with influenza A virus and cloned by limiting dilution. T-cells from 8 colonies were stimulated with influenza A (HA units/ml), influenza B (5HA units/ml) GAT (500μg/ml), TGAL (1 mg/ml), T. Tox (0.1 LF/ml) and KLH (80μg/ml) together with $25x10^3$ autologous irradated PBL. Stimulation was assessed by the incorporation of ^{3}H-Thymidine in a 72 hour assay.

*Underline indicates cpm 5-fold or greater over background

Table IV

TLC 53 was prepared as described in legend to Table II. This table shows the results of experiments in which $5x10^3$ T-cells from TLC 53 were cultured with or without influenza A, influenza B, GAT, TGAL, T.Tox and KLH. $25x10^3$ autologous irradiated (2500 Rads) PBL (PBL(IR)), $20x10^3$ autologous irradiated sheep erythrocyte positive (E^+) cells (E^+(IR)), $5x10^3$ autologous irradiated E^- (E^-(IR)) and $20x10^3$ (E^+ (IR)) and $5x10^3$ (E^- (IR)) cells together were used as a source of antigen presenting cells (APC). The different fractions of APC were added to $5x10^3$ TLC (clone 53) together with antigen and the incorporation of ^{3}H-thymidine measured at 72 hours. The stimulation of the APC fractions both irradiated and unirradiated by the antigens alone is shown in the table. Positive responses are underlined.
* Response of TLC 53 to TCGF control.

Table V

Unfractionated PBL from a high responder were stimulated with influenza virus A and cloned by limiting dilution. Cells from 8 TLCs were stimulated with influenza A (5HA units/ml), influenza B(5HA units/ml) GAT (500 μg/ml), TGAL (1mg/ml), T. Tox (0.1 LF/ml and KLH (80μg/ml) together with $5x10^3$ autologous irradiated E^- cells. Stimulation was assessed by ^{3}H-Thymidine incorporation in a 72 hour assay.

Table VI

TLCs 6, 26, 37 and 71 were stimulated with influenza A (5HA/ml), influenza B (5HA/ml), haemagglutinin (HA, 0.1μg/ml), neuraminidase (NA, $5x10^{-4}$ vol %) and matrix protein (MP, 0.1μg/ml) together with $5x10^3$ autologous irradiated E^- cells. Stimulation was assessed by ^{3}H-thymidine incorporation in a 72 hour assay. Positive responses are underlined.

Legend to Figures

Figure 1. Kinetics of the TLC proliferation to influenza virus, strain A. Unfractionated PBL from a high responder were stimulated with influenza A virus and cloned by limiting dilution. Cells ($5x10^3$) from 8 TLCs were restimulated with influenza A together with $25x10^3$ autologous irradiated PBL (o--o). Stimulation was assessed by the incorporation of ^{3}H-thymidine at 12, 24, 36, 48, 60, 72, 96 and 144 hours after the initiation of the culture. Control responses of the colonies to TCGF (+--+) and medium (•--•) are shown for each clone. Each point represents the mean of triplicate cultures.

TABLE I

Distribution of responsiveness of influenza specific T cell clones

		Proliferative Response (cpm)	Number of clones	Reactivity Index[a] A (0-0.5)	B (0.5-1.0)	C (>1.0)
Group	I	1-500	27	25	1	1
	II	500-3000	27	26	1	0
	III	3000-10,000	27	4	3	20
	IV	>10,000	15	0	0	15

TABLE II

Distribution of influenza TLC into high, intermediate and low responders

Clone Number	Proliferative response (cpm ± SEM) of TLC					Reactivity Index
	+PBL(IR) +Flu	+Flu	+PBL(IR)	+TCGF	Medium	
50	222(58)	10(2)	24(3)	8492(1126)	39(14)	0.03
76	219(38)	11(1)	15(4)	4870(218)	15(2)	0.04
18	1280(242)	13(1)	34(3)	4427(689)	17(1)	0.29
77	2105(416)	14(1)	18(5)	5261(754)	13(3)	0.40
69	4136(1068)	14(1)	112(26)	3576(747)	33(5)	1.16
88	4774(365)	9(3)	26(7)	3753(847)	13(1)	1.27
6	35149(2394)	23(5)	134(44)	10845(152)	17(5)	3.33
24	12005(1883)	18(2)	118(43)	2078(32)	20(5)	5.78
26	14243(3414)	15(2)	32(6)	3637(954)	17(5)	3.92
37	24534(5897)	16(4)	639(163)	7142(404)	5(1)	3.44
53	27051(4555)	13(1)	1549(335)	7670(1240)	17(2)	3.53
71	32689(7629)	11(4)	2248(392)	7703(490)	37(3)	4.24
72	15567(3059)	18(1)	42(11)	1375(202)	25(1)	11.32

Controls:

PBL(IR)			
+Flu	+PWM	+PHA	Alone
11(3)	16(3)	16(3)	8(2)

TABLE III

Response of influenza A virus induced TLC to specific and unrelated antigens

Antigen	Proliferative response (cpm) of TLC							
	Clone Number:							
	6	24	26	37	53	71	72	50
Influenza A (5HA/ml)	13707*	9549	11935	11049	8130	9475	3191	67
Influenza B (5HA/ml)	120	33	45	116	59	101	32	55
GAT (500μg/ml)	19	25	25	67	22	68	40	65
TGAL (1mg/ml)	26	18	18	147	33	58	122	298
T. Toxoid (0.1 LF/ml)	424	200	730	985	610	1593	37	64
KLH (80μg/ml)	61	26	21	68	47	196	42	23
Medium	21	10	15	14	19	36	12	18
TCGF	2197	689	1266	1143	1213	2011	363	1476

TABLE IV

Effect of the antigen presenting cell population on clone specificity

Proliferative response of TLC 53 (cpm ± SEM)

Cells		Antigen							Mitogen
TLC	APC	Flu A	Flu B	GAT	TGAL	T. Tox.	KLH	Medium	PWM
-	PBL	39(6)	38(14)	17(2)	25(1)	18(4)	184(144)	9(3)	601(135)
-	PBL(IR)	19(2)	21(5)	20(4)	9(1)	16(3)	12(2)	15(2)	942(72)
53	PBL(IR)	8310(553)	59(27)	22(6)	33(15)	610(117)	47(10)	19(5)	-
-	E^+	17(3)	27(9)	20(4)	15(3)	17(4)	15(4)	17(4)	108(34)
-	E^+(IR)	21(4)	15(2)	19(2)	18(3)	19(1)	13(1)	19(2)	21(3)
53	E^+(IR)	32(10)	24(3)	49(3)	10(0)	39(8)	37(1)	22(2)	-
-	E^-	9(1)	30(2)	12(0)	25(5)	21(6)	36(6)	13(3)	23(1)
-	E^-(IR)	11(1)	23(6)	18(2)	17(5)	13(4)	24(4)	12(1)	38(4)
53	E^-(IR)	5542(590)	26(7)	51(47)	25(1)	59(14)	43(12)	19(3)	-
-	E^++E^-	197(33)	68(18)	24(5)	65(8)	110(29)	53(7)	45(5)	4163(320)
-	E^++E^-(IR)	13(2)	21(5)	15(3)	17(2)	15(4)	15(1)	19(5)	1683(132)
53	E^++E^-(IR)	8384(991)	86(6)	24(16)	48(13)	761(195)	37(3)	11(3)	-
53	-	17(9)	24(4)	16(0)	10(4)	25(7)	33(7)	28(2)	1366(94)*

TABLE V

Antigenic specificity of the T lymphocyte clones

Antigen	Proliferative response (cpm ± SEM) of TLC Clone Number: 6	24	26	37	53	71	72	50
Influenza A (5HA/ml)	12661(239)	7442(160)	5658(830)	6306(590)	5542(590)	2151(332)	1624(128)	23(3)
Influenza B (5HA/ml)	171(135)	35(27)	19(7)	289(57)	26(7)	18(8)	16(2)	50(36)
GAT (500μg/ml)	82(64)	14(3)	13(1)	41(27)	51(47)	10(12)	16(4)	26(0)
TGAL (1mg/ml)	16(2)	62(2)	26(14)	20(0)	25(1)	30(4)	73(57)	11(1)
T.Toxoid (0.1 LF/ml)	50(4)	10(2)	37(7)	57(25)	59(14)	37(11)	38(2)	124(34)
KLH (50μg/ml)	29(11)	19(4)	6(0)	46(9)	43(12)	420(188)	43(10)	39(3)
Medium	22(4)	17(4)	37(29)	15(1)	19(3)	27(1)	18(3)	202(184)
TCGF	1943(53)	701(141)	1271(190)	1339(281)	1244(74)	2117(490)	404(8)	1872(270)

TABLE VI

Specificity of TLC responses to viral subcomponents

Proliferative response (cpm ± SEM)

Antigen	Clone Number: 6	26	37	71
Influenza A (5 HA/ml)	9843(504)	7812(1728)	18191(479)	8570(2018)
Influenza B (5 HA/ml)	45(8)	16(1)	20(2)	24(8)
HA (0.1 μg/ml)	70(30)	19(1)	41(5)	28(14)
NA ($5x10^{-4}$vol%)	21(1)	3680(344)	19(5)	5291(1249)
MP (0.1 μg/ml)	4132(751)	19(2)	9752(1677)	27(8)
Medium	146(45)	23(3)	30(6)	14(3)
TCGF	4725(395)	5039(769)	10987(1159)	2741(507)

TABLE VII

FACS analysis of T-lymphocyte clones

Monoclonal Antibody	Clone Number: 6*	37*
HLA framework	+++	+++
DR	+++	+++
OKT3	++	++
OKT4	+	+
OKT6	-	-
OKT8	-	-
Leu-1	+++	±
Leu 2A	-	-
Leu 3A	++	+++
sIg	-	-

*Each TLC formed >95% E-rosettes.

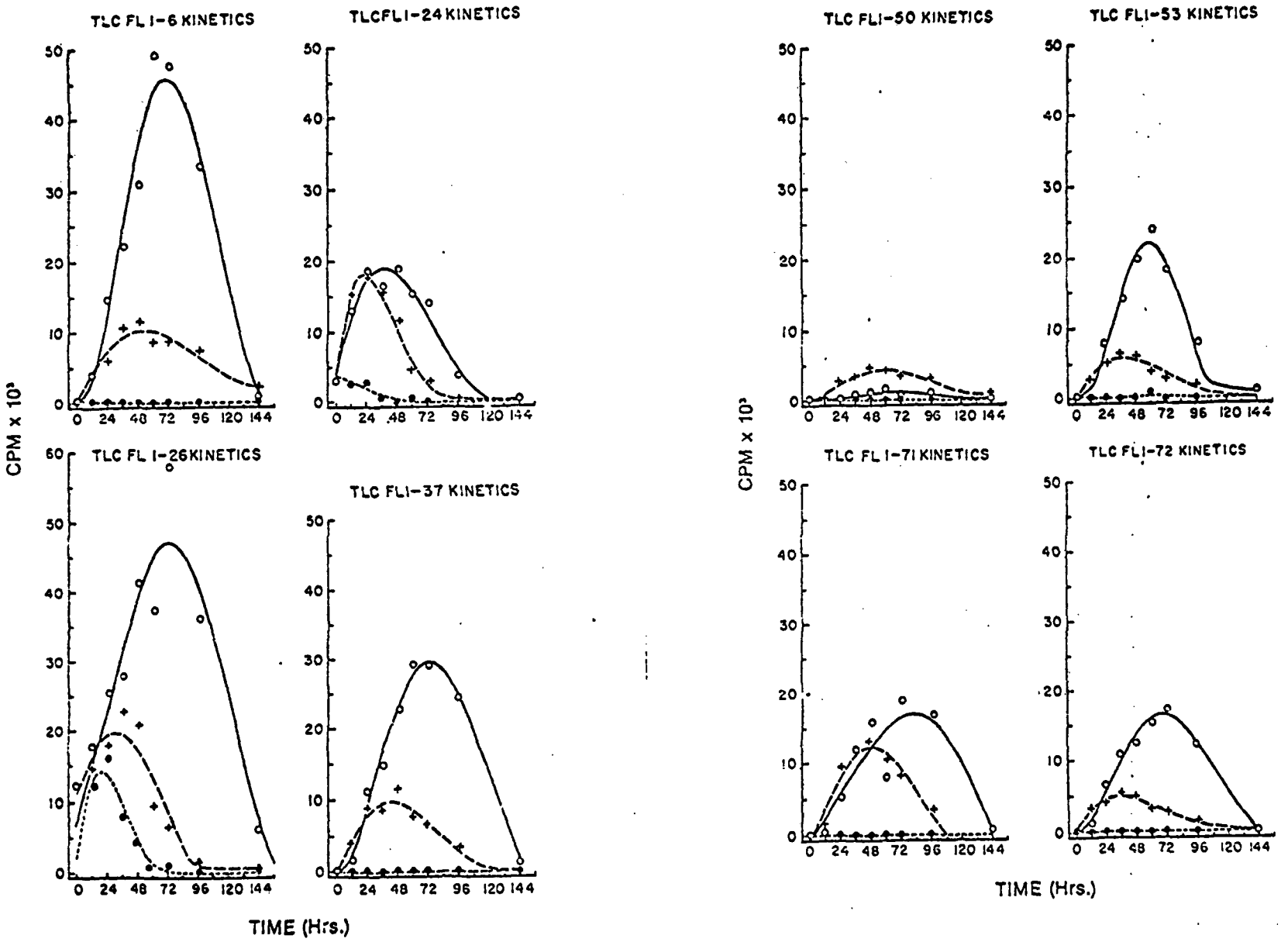
TLC FL I-6 KINETICS
TLCFLI-24 KINETICS
TLC FL I-26 KINETICS
TLC FLI-37 KINETICS
TLC FLI-50 KINETICS
TLC FLI-53 KINETICS
TLC FL I-71 KINETICS
TLC FLI-72 KINETICS
CPM x 10³
TIME (Hrs.)
0 24 48 72 96 120 144
0 10 20 30 40 50 60

Figure 1.

Clin. Exp. Immunol. (1981) 43, 517–525

ATTACHMENT NO. 2
CLINICAL AND EXPERIMENTAL IMMUNOLOGY
(1981) 43. 517-525

Induction of human antigen-specific suppressor factors *in vitro*

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Accepted for publication 12 September 1980

SUMMARY

Based on methods used for the *in vitro* induction of antigen-specific suppressor cells in the mouse, we have cultured Ficoll–Isopaque-separated human blood cells with high dose of antigen (100 μg/ml) in Marbrook culture vessels for 4 days. The resulting cells, when further recultured for 24 hr with a low dose of antigen (1 μg/ml), released into the supernatant material, termed 'suppressor factor', which inhibited, in an antigen-specific manner, the antibody response of mouse spleen cells in culture. The suppressor factor was analysed using immunoabsorbents, and was bound to and eluted from specific antigen, concanavalin A and lentil lectin, anti-human Ia antibodies, and anti-mouse suppressor factor antibodies, but was not bound to antibodies against human IgG.

INTRODUCTION

The importance of antigen-specific suppressor cells in immune regulation is well established and their involvement in many human diseases has long been suspected (reviewed by Gershon, 1974; McMichael & McDevitt, 1977; Waldmann & Broder, 1977; Siegal, 1978). Until now, only non-antigen-specific suppression, induced by mitogens such as concanavalin A, has usually been assayed. Thus assays of antigen-specific functions of human T cells are of interest, and should permit a deeper understanding of many disease states. This would supplement the limited information available from measuring the numbers of circulating T cells, or the mitogenic stimulation of T cells, where subsets of T cells cannot be distinguished.

In the mouse, T cells mediating help, suppression or cytotoxicity are distinct cells, clearly differentiated by surface phenotypic markers (reviewed by Cantor & Boyse, 1976; Feldmann *et al.*, 1977a). Surface structures characteristic of human T cells have recently been described (Moretta *et al.*, 1977; Strelkauskas *et al.*, 1978; Reinherz *et al.*, 1979) and their evaluation in functional tests started (Moretta *et al.*, 1977; Balthieux *et al.*, 1979).

There is much evidence that antigen-specific murine suppressor cells act via secreted mediator molecules, suppressor factors (Kontiainen & Feldmann, 1977; Tada, Taniguchi & Okumura, 1977). These are antigen-specific and inhibit helper cells of the same antigenic specificity. Serological analysis has shown that these factors carry major histocompatibility complex (MHC) coded determinants but lack immunoglobulin Fc determinants, and are characterized by factor 'constant' and 'variable' region determinants defined by rabbit and mouse anti-factor antisera (Kontiainen & Feldmann, 1978, 1979; Feldmann *et al.*, 1976, 1977b). The secreted antigen-specific suppressor factors are not strain- or species-restricted, e.g. murine suppressor factors can be generated in one

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strain of mice and assayed on another, and monkey suppressor factors assayed on human peripheral cells *in vitro* (Koskiaanen & Feldmann, 1977; Family, Koskiaanen & Lehner, 1979). The lack of species restriction has also been shown with human (Kantor & Feldmann, 1979) and mouse (Family, Koskiaanen & Lehner, 1980) antigen-specific helper factors, but is not expected when obtained with factor molecules extracted from cells (e.g. Lada *et al.*, 1977).

As the importance of antigen-specific suppression is well documented, we decided to see whether antigen-specific suppressor factors could be generated from human peripheral blood cells in tissue culture. We here describe the generation of such factors using protein or polypeptide antigens, their effects on antibody responses *in vitro* and their structural characteristics.

MATERIALS AND METHODS

Human peripheral blood (PBL = mononuclear cells). Peripheral blood (20–30 ml) was collected from normal healthy laboratory personnel into heparinized syringes, or buffy coat cells from a 400-ml unit of freshly drawn heparinized blood from the Finnish Red Cross Blood Transfusion Centre were used. The isolation of mononuclear cells was started within 1 hr (laboratory personnel) or 4 hr (buffy coat cells) after the blood was collected. Peripheral blood was first diluted 1+3, 4 buffy coat cells 1+4–5 in phosphate-buffered saline (PBS, pH 7.2) and 40 ml of the dilution added to round-bottomed 50-ml centrifugation tubes. Ten millilitres of Ficoll–Isopaque or Ficoll–Urografin (sp. gr. 1.077, Boyum, 1968) was carefully layered at the bottom, and the tubes spun at 1,000 *g* for 30 min at room temperature using Sorvall RC-3 or an MSE centrifuge. The cells at the interface were collected, washed twice in PBS, and the numbers of viable cells assessed using trypan blue exclusion. The yield of mononuclear cells ranged between 0.5 to 2 × 10^7 per ml of blood. Cytocentrifuge smears were occasionally prepared after Ficoll–Isopaque separation. After Ficoll–Isopaque separation about 90% of the cells were morphologically small lymphocytes, as compared to about 50% before Ficoll–Isopaque separation. PBL from laboratory personnel were obtained voluntarily and with informed consent. Buffy coats are routinely separated from the donated blood at the Finnish Red Cross Blood Transfusion Centre and normally used for interferon production.

Antigens. The antigens used were keyhole limpet haemocyanin (KLH), chicken gamma-globulin (CGG), a terpolymer of L-glutamic acid[60]-L-alanine[30]-L-tyrosine[10] (GAT, Miles Yeda Ltd.), another synthetic polypeptide poly(L-tyrosine-L-glutamic acid)-poly(D,L-alanine)-poly(L-lysine) (abbreviated (T,G)-A--L) and their dinitro- (DNP) or trinitro- (TNP) phenylated derivatives. TNP-KLH had eight and DNP-CGG seven groups of TNP or DNP per 10^5 daltons, and DNP-GAT and DNP-(T,G)-A--L two groups of DNP per 10^5 daltons. KLH was kindly provided by Professor M. B. Rittenberg, University of Oregon, Portland, USA, and trinitrophenylated as previously described (Rittenberg & Amkraut, 1966). CGG was prepared by ammonium sulphate precipitation from normal chicken serum, and dinitrophenylated as described by Eisen (1964). (T,G)-A--L was kindly donated by Professor Edna Mozes, Department of Chemical Immunology, Weizmann Institute, Rehovot, Israel. GAT and (T,G)-A--L were dinitrophenylated as previously described (Howie *et al.*, 1977).

Animals. Because of the lack of a reproducible human B cell response system, suppressor factors were tested using spleen cells from normal CBA or B10.SeSn mice or from CBA mice immunized with KLH, TNP-KLH or DNP-CGG *in vivo*. The TNP-KLH-immunized mice received three intraperitoneal injections of 100 μg of TNP-KLH in Freund's complete adjuvant at weekly intervals followed by three intraperitoneal injections of 100 μg of TNP-KLH in saline together with 10^9 *Bordetella pertussis* organisms at weekly intervals. DNP-CGG-primed mice received three weekly injections of 100 μg/ml DNP-CGG absorbed onto bentonite (Rittenberg & Pratt, 1969), and KLH-primed mice three injections of 100 μg/ml of KLH in Freund's complete adjuvant. The spleens were used 2 weeks to 2 months after the last antigen injection. CBA mice bred at the Department of Bacteriology and Immunology, University of Helsinki, were mainly used. Testing of GAT or (T,G)-A--L suppression was made using B10 mice from the ICRF breeding unit. The unimmunized mice were about 3 months of age when used, immune mice were 6–8 months old when used.

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Tissue culture conditions. The tissue culture medium used was RPMI 1640 supplemented with fetal calf serum (FCS, Flow), penicillin (100 iu/ml) and streptomycin (100 μg/ml). Batches of FCS were tested for the ability to support induction of immune helper cells and cooperative cultures and the selected batches were also used for cultures of human cells. The medium in the outer compartment of the Marbrook flasks was bicarbonate buffered and contained 5×10^{-5} M 2-ME. The cells in the inner compartment of Marbrook flasks were in Eagle's modified buffered medium. All the cultures were performed at 37°C in a humidified atmosphere of 10% CO_2 in air.

Induction of suppressor cells and factors. The induction of suppressor cells and factors was essentially as previously described with murine cells (Kontiainen & Feldmann, 1976, 1978). Briefly, 5×10^{7}–10^{8} PBL mononuclear cells were cultured with 100 μg/ml of KLH, GAT or (T,G)-A--L in the inner compartment of Marbrook flasks for 4 days *in vitro*. After 4 days of culture the cells were harvested, washed and the numbers of viable cells counted using trypan blue exclusion. The average yields of viable cells after the 4 day culture were 40–80% with higher recoveries if the cell density was kept lower. The cells adjusted to 2×10^{6} viable cells/ml were further cultured for 24–48 hr in Marbrook flasks together with 1 μg/ml of the antigen used during the first 4 day culture. After the 24–48-hr culture the supernatants were harvested, spun down at 1500 r.p.m. for 10 min, filtered through Millipore filters, adjusted and stored at −20°C. These supernatants were termed 'suppressor factors' (SF) and the specificity (antigen used in induction) indicated as SF_{KLH}, SF_{GAT} etc.

Assay of suppressor factor activity. Suppressor factors were assayed on normal spleen cells using either *in vitro*- or *in vivo*-primed helper cells or spleen cells from mice primed *in vivo* with DNP-KLH. Helper cells specific for KLH, GAT or (T,G)-A--L were primed *in vitro* as previously described (Kontiainen & Feldmann, 1973; Howe *et al.*, 1977; McDougall & Gordon, 1977). Briefly, 5×10^{6} spleen cells were incubated for 4 days *in vitro* with 1 μg/ml of KLH, GAT or (T,G)-A--L in Marbrook flasks. After 4 days in culture the cells were harvested and the numbers of viable cells assessed. These cells were termed HC. Three hundred thousand HC were added to 2×10^{6} normal spleen cells together with 1 μg/ml of the appropriate antigen, DNP-KLH, DNP-GAT or DNP-(T,G)-A--L in the presence or absence of the SF. When *in vivo*-KLH-primed helper cells were used, spleen cells from KLH-immunized mice were filtered through nylon wool columns (Julius & Greenberg, 1973) and 3×10^{6} of the passed cells added to 2×10^{6} anti-Thy 1.2 monoclonal antibody (Thy-1.2 TD5 kindly provided by Dr Phil Baker) and complement treated (Feldmann *et al.*, 1977) spleen cells from DNP-(T,G) immunized mice together with 0.2 μg/ml of DNP-KLH. When DNP-KLH-primed mice were used for secondary cultures, 3×10^{6} spleen cells were cultured with 0.1 μg/ml DNP-KLH in the presence or absence of SF. In all the cultures were stimulated in triplicate. In cultures with *in vitro*-primed HC were assayed only 4 of the cooperative cellular interactions: DNP-KLH response by *in vivo*-DNP-KLH-primed spleen cells or by KLH-primed and DNP-(T,G)-primed B cells on day 5 or 6 of the culture.

Assay of antibody-forming cells (AFC). The numbers of anti-DNP AFC in cultures were assayed as previously described (Kontiainen & Feldmann, 1973, 1978) using DNP-Lab coupled sheep red blood cells. With *in vitro*-primed HC only IgM anti-DNP antibody-forming cells were detected. With *in vivo*-DNP-KLH-primed spleen cells or KLH-primed HC together with DNP-primed B cells yielded both IgM and IgM anti-DNP antibody-forming cells. The IgM anti-DNP AFC were detected using goat anti-μ to block the IgM producers and sheep anti-γ to block IgM producing cells. The results were expressed as anti-DNP AFC per 10^{6} cultured cells.

Immunosorbents and their use. KLH, anti-phenylated bovine serum albumin (NIP-BSA) and anti-μ (BS-μ) were coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) beads following the manufacturer's instructions. For 1 g dry weight of beads 5 mg of the above antigens were used. The rabbit anti-suppressor and anti-helper factor antisera as well as the anti-human immunoglobulin preparation, swine anti-human IgG (anti-heavy and anti-light chains, batch LC 25, Orion Diagnostica, Helsinki, Finland) were coupled using 0.5 ml of antiserum per 1 g of beads. The preparation of rabbit anti-factor antisera has been previously described (Kontiainen & Feldmann, 1979). Concanavalin A (Con A) Sepharose 4B and Staph Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). The absorptions (5 ml of 50% ml of coupled beads) were for 2 hr at room temperature on a rotating mixer after which

the supernatant with non-absorbed material (filtrate) was taken off. The beads were washed four times with PBS to remove any unbound material and then eluted using glycine-HCl buffer, pH 2.4. The eluates were dialysed against saline at 4°C overnight. The absorbed and eluted materials were Millipore filtered, aliquoted and stored at −20°C.

RESULTS

Effects of human SF on primary and secondary antibody responses in vitro

Mouse antigen-specific SF can eliminate both primary and secondary antibody responses *in vitro*. Thus the effects on primary and secondary responses of human SF were ascertained. The results (Table 1 and Fig. 1) using human KLH, (T,G)-A--L or GAT-specific suppressor factors indicate that human SF abrogate primary and secondary antibody responses by mouse spleen cells efficiently.

Table 1. Specificity of human suppressor factors

Stimulus				Response anti-DNP AFC (10^3 ± S.E.)	
Helper cells	Spleen cells	Antigen	Suppressor in SF	IgM	IgG
Expt A					
	+	TNP-KLH		22 ± 6	44 ± 44
HC_{KLH}	+	TNP-KLH		[illegible] ± 43	1,522 ± 238
HC_{KLH}	+	TNP-KLH	$HuSF_{KLH}$ 5%	250 ± 48	556 ± 80
HC_{KLH}	+	TNP-KLH	$HuSF_{KLH}$ 0.5%	294 ± 56	778 ± 118
HC_{KLH}	+	TNP-KLH	MSF_{KLH} 5	256 ± 22	733 ± 102
HC_{KLH}	+	TNP-KLH	MSF_{KLH} 0.5	456 ± 87	1,200 ± 133
Expt B					
	+	DNP-(T,G)-A--L		18 ± 10	
$HC_{(T,G)-A--L}$	+	DNP-(T,G)-A--L		90 ± 14	
$HC_{(T,G)-A--L}$	+	DNP-(T,G)-A--L	$HuSF_{(T,G)-A--L}$	11 ± 6	
$HC_{(T,G)-A--L}$	+	DNP-(T,G)-A--L	$HuSF_{GAT}$	71 ± 16	
Expt C					
	+	DNP-GAT		10 ± 5	
HC_{GAT}	+	DNP-GAT		98 ± 21	
HC_{GAT}	+	DNP-GAT	$HuSF_{GAT}$	22 ± 11	
HC_{GAT}	+	DNP-GAT	$HuSF_{(T,G)-A--L}$	120 ± 22	

In Expt A, human KLH-specific suppressor factor ($HuSF_{KLH}$) or B10.A (5R) SF_{KLH} (MSF_{KLH}) at a final concentration of 5 or 0.5% was added to 3×10^6 spleen cells from mice primed with TNP-KLH and cultured without added antigen (line 1, background) or with 0.05 μg/well of TNP-KLH in the absence (response, line 2) or presence of SF_{KLH} (lines 3–6). In other experiments SF were added to the mixture of 3×10^5 nylon wool-passed spleen cells from CBA mice immunized three times with 100 μg of KLH in Freund's complete adjuvant (helper cells, HC) and 3×10^6 anti-Thy 1.2-treated spleen cells from mice immunized three times with DNP-CGG on bentonite (B cells). The stimulation was with 0.1 μg/ml of TNP-KLH ml either in the absence of HC or with added HC in the absence of SF or with SF added. IgM and IgG anti-DNP AFC were assayed on day 5 or 6. All cultures were started in triplicates.

In Expt B, 3×10^5 $HC_{(T,G)-A--L}$ of B10 origin were added to 3×10^6 normal B10 cells together with 1 μg/ml of DNP-(T,G)-A--L in the presence or absence of 5% (final concentration) of human $SF_{(T,G)-A--L}$ ($HuSF_{(T,G)-A--L}$) or SF_{GAT} ($HuSF_{GAT}$).

In Expt C, 3×10^5 HC_{GAT} of CBA origin were added to 3×10^6 normal CBA spleen cells together with 1 μg/ml of DNP-GAT in the presence or absence 5% (final concentration) of human SF_{GAT} or $SF_{(T,G)-A--L}$. The same SF preparations as in Expt B were used.

In Expt B and C, IgM anti-DNP AFC responses were measured on day 4 of the culture. All the cultures were started in triplicates. Three other experiments gave similar results.

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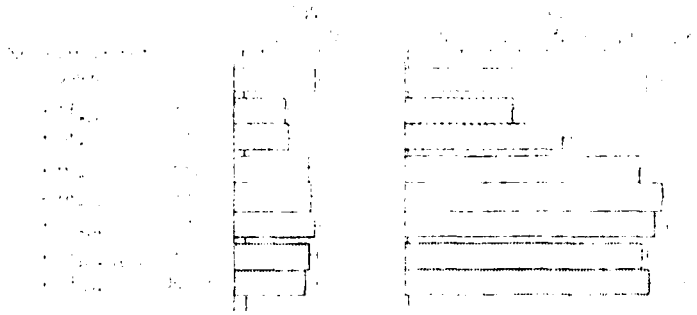

Fig. 1. Specificity of human SI$_{KLH}$. Human SI$_{KLH}$ or SI$_{pork\ insulin}$ at a final concentration of [illegible] or 0.2 [illegible] was added to 3 × 10^6 spleen cells from TNP KLH immunized CBA mice (spleen cells were 3 × 10^6/ml). TNP KLH was another control. KLH specific helper factor (HF$_{KLH}$) induced from the mouse lymph node cells of the same donor was used to induce SI$_{KLH}$ and SI$_{pork\ insulin}$ was used at final concentration of [illegible] or [illegible]. The dose of KLH used to induce HF$_{KLH}$ was 1 μg/ml of KLH (as opposed to 100 μg/ml for the induction of SI$_{KLH}$). The cultures were started in triplicate. IgM and IgG anti-DNP AFC were assayed on day 6 of culture. Solid lines are the background response by TNP KLH primed spleen cells without adding TNP KLH.

Table 2. Characteristics of human SI$_{KLH}$

Stimulus			Response: anti-DNP AFC (10^6 ± S.E.)	
TNP-KLH primed spl.	TNP KLH	Suppression SI$_{KLH}$	IgM	IgG
Expt A				
+			6 ± 6	56 ± 56
+	+		322 ± 20	3,333 ± 259
+	+	+	83 ± 28	226 ± 111
+	+	+ abs. KLH	272 ± 6	2,722 ± 233
+	+	+ ads. KLH	78 ± 6	833 ± 355
+	+	+ abs. NIP-BSA	94 ± 20	667 ± 255
+	+	+ ads. KLH	278 ± 26	2,944 ± 294
Expt B				
+			28 ± 6	22 ± 2
+	+		1,389 ± 100	4,833 ± 536
+	+	+	639 ± 47	1,889 ± 511
+	+	+ abs. rHuIgG	794 ± 115	2,333 ± 646
+	+	+ abs. Con A	1,510 ± 192	4,667 ± 874
+	+	+ abs. lentil lectin	1,494 ± 86	4,089 ± 588
Expt C				
+			28 ± 20	0
+	+		1,700 ± 192	11,556 ± 964
+	+	+	1,564 ± 200	3,889 ± 222
+	+	+ abs. RsHuIgG	1,733 ± 200	10,778 ± 721

In each experiment, 3 × 10^6 spleen cells from mice primed with TNP KLH were cultured without TNP KLH (background, line 1 in each experiment) or with 0.02 μg/ml of TNP KLH (in the absence of response, line 2 in each experiment) or presence of SI$_{KLH}$ at 5% final concentration (either unabsorbed or absorbed as shown). Anti-DNP AFC were assayed on day 6 of the culture. All the cultures were started in triplicates.

Three to four other experiments at each point gave similar results.

a comparable degree to mouse suppressor factors (Table 1, Expt A). The degree of suppression ranges from 40–100% and is comparable for both IgM and IgG responses. The extreme specificity of the suppression is shown by the lack of effect of GAT-induced factors on TNP-GAT-primed spl, TNP-GAT-induced factors on GAT-specific help (Table 1), and by the lack of effect of insulin-specific suppressor factors on secondary TNP-KLH responses (Expt B). The specificity was further confirmed by the immunoadsorption studies (Table 2) as adsorption with KLH but not with NIP-BSA abolishes the suppressive activity of human SI_{KLH}. The requirement for high TNP concentration in the induction phase is also ascertained as supernatants from human cells cultured with Lucentis KLH which contain HF (Koottaanen & Feldmann, 1979) have no suppressive effect.

Characterization of human SI with murine and rabbit antisera

Because of the minute concentrations of factors and certain cell factor properties, immunoadsorbent analysis has proven to be the most convenient type of characterization (reviewed by Feldmann & Okumura, 1979; Feldmann & Koottaanen, 1980). For the analysis of the specificity of the SI activity from human cells was performed, as described in the Materials and Methods section. Results shown in Table 2 indicate that the functional antigen-specificity of human suppressor factors is demonstrated by the ability to be matched by binding to KLH but not to NIP-BSA. The similar characteristics of binding to GAT, lentil lectin and rabbit anti-human Ig but not to anti-human IgG.

These studies have been performed also with SI_{TNP} and insulin, and with anti-Ia antisera, including one monoclonal reagent with analogous results (not shown).

Reaction of human SI with murine and rabbit antisera against factors

Mouse helper and suppressor factors have also been characterized by immunoadsorption with rabbit antisera against factors. The latter were not specific for mouse strain or antigen specificity but distinguished accurately between HF and SF. This led to the concept of constant and variable regions on factors (Koottaanen & Feldmann, 1979). Because human SF reacted with anti-mouse helper

Table 3. The effects of adsorptions with anti-suppressor factor antisera on the activity of human SI_{KLH}

Stimulus			Response: anti DNP AFC/10⁶ spl	
TNP-KLH-primed spl	TNP-KLH	Suppressor: SI_{KLH}	IgM	IgG
Expt A				
+			26 ± 6	32 ± 22
+	+		1,862 ± 1,001	4,533 ± 736
+	+	+	630 ± 47	1,898 ± 514
+	+	+ abs. sSF 107	1,172 ± 89	4,089 ± 1,157
+	+	+ abs. sSF 422	560 ± 68	2,861 ± 791
+	+	+ abs. sHF 122	58 ± 29	1,178 ± 446
Expt B				
+			6 ± 6	0
+	+		1,606 ± 89	2,311 ± 97
+	+	+	778 ± 31	2,622 ± 270
+	+	+ abs. sSF 201	1,828 ± 204	2,867 ± 444
+	+	+ abs. sSF 124	1,106 ± 839	6,667 ± 839

See legend to Table 2 for details.

The adsorptions were with rabbit anti-suppressor factor antisera derived from rabbits 107, 124, 201 and 422 immunized with purified SI_{KLH} or with anti-helper factor antisera derived from rabbit 122 immunized with purified HF_{KLH}. For immunizations and characterization of the antisera see Koottaanen & Feldmann (1979). The adsorptions of Expt A tested in the same Expt as Expt B in Table 2.

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[illegible] T and B cells. It was possible that [illegible] suppressor [illegible] associated with the [illegible] suppressor [illegible] abundant [illegible] factor. Thus factor SI [illegible] with [illegible] SI. [illegible] show that this is indeed the case and the [illegible] specificity is due to shared [illegible] SI's substrate's results with [illegible] which [illegible] specific antibodies (Kontiainen & Feldmann [illegible]).

DISCUSSION

There have been attempts to study antigen-specific functions of human T cells and [illegible] basis. Thus the need of macrophages in the stimulation of peripheral blood T cells with antigens or the induction of antigen-specific helper factor production has been documented (Rosenberg & Barnes, 1978; Mudgwar, Yams & Geha, 1978; Ree *et al.*, 1979) and [illegible] antigen-specific helper factor production to synthetic polypeptide antigens [illegible] (1979). There is one report of antigen-specific suppressor cells and factors in human [illegible] B cells in the study by de Haan *et al.* (1978, 1979).

In this paper antigen-specific suppressor factors generated from [illegible] human peripheral blood mononuclear cells were characterized. These were antigen specific in [illegible] and thus resembled the primate T cell [illegible] (1979) and murine [illegible] *et al.*, 1977; Benacerraf & Dorf, 1976; Kontiainen & Feldmann, 1978; [illegible]).

There was binding to Con A and lentil lectin coincident with the [illegible] Reaction of rat or anti-rat antiserum with SI is analogous to studies in the mouse (Benacerraf & Dorf, 1976; Kontiainen & Feldmann, 1978; Tada & Okumura, 1979), although the idiotypic specificity association is not possible with the anti-human antiserum. The lack of reactivity with anti-Ig is again comparable (Tada *et al.*, 1977; Kontiainen & Feldmann, 1978), although recent studies have implicated Ig variable region determinants in the combining site (reviewed in Tada & Okumura, 1979).

The lack of species restriction is comparable to results with other antigen-specific human and murine factors acting across species barriers (Kantor & Feldmann, 1979; [illegible]) and implies conservation of structure (Kontiainen & Feldmann, 1979).

These results were not easy to reconcile with reports of mouse strain genetic restriction of suppressor cell extracts (reviewed by Feldmann & Kontiainen, 1979), but confirm the other reports that suppressor cell extracts and supernatants are not identical in the mouse.

Although the enumeration of T and B cells and their stimulation with antigens gives useful information in many clinical conditions, they do not reveal whether responses to specific antigens may be affected.

Although many of the antigens in potential autoimmune T and B pathogenesis of human disease are still undefined, there are purified antigens, such as the tuberculin preparations, the [illegible] purified DNA or purified polysaccharide or protein antigens, [illegible] where [illegible] generation of disease or its prevention cannot be disputed. That there is [illegible] tests which would [illegible] of similar [illegible] of distinct T and B cell subsets with antigens using the patient's own blood cells, or [illegible] induction of suppressor [illegible] induction of B cells in antibody production. This may lead to [illegible] diagnosis, and it is conceivable that immunoregulatory molecules such as antigen-specific suppressor factors may eventually be [illegible] to allow [illegible] elimination of [illegible] in some respects.

[illegible] supported by the [illegible] Wellcome Medical [illegible] Science [illegible] [illegible] Suzanne [illegible] preparing the manuscript [illegible] Mr [illegible] Mr [illegible] Mr [illegible] Con A [illegible] Phil Lake [illegible] Dr R. Jones [illegible] preparation of the anti-human [illegible] Dr [illegible] Blood Transfusion [illegible] human [illegible]

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ATTACHMENT NO. 3: Presented at the "Third International Workshop on Alloantigenic Systems in the Rat" - June 1980, Philadelphia, PA.

INHIBITION OF MIXED LYMPHOCYTE RESPONSE BY MONOCLONAL ANTIBODIES SPECIFIC FOR RAT LYMPHOCYTE SUBSETS

J.A. Bash, S. Shapiro, L.E. Adams and J.N. Woody. Immunologic Oncology Division, Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C. 20007.

Monoclonal antibodies specific for rat lymphocyte membrane antigens have been produced by fusing mouse myeloma cells and spleen cells from mice immunized with rat cell membrane antigens (Williams et al. Cell 12:663, 1977). These reagents have previously been shown to be useful in defining functional subsets of cells involved in the generation of mixed lymphocyte responses (MLR, Webb, et al. Nature 282:841, 1979). In the present study antibody produced by clones W3/13 and W3/25 (anti-T cell) and OX3 (anti-Ia glycoprotein) were added at the start of culture of F344 ($RT1^{l}$) responder spleen cells or peripheral blood lymphocytes with BN ($RT1^{n}$) or DA ($RT1^{av1}$) stimulator spleen cells (2000 R irradiated). MLR responses at 120 hr were determined by ^{3}H-thymidine incorporation (18 hr pulse). Responder cells were simultaneously cultured with phytohemagglutinin (PHA) in the presence of antibodies and assayed for ^{3}H-thymidine incorporation at the end of 72 hours.

In agreement with the results of Webb et al. (Nature 282:841, 1979) W3/25 antibody was highly inhibitory to MLRs (100% inhibition at 50 ng/ml) while W3/13 failed to inhibit even at 50 mg/ml. Interestingly, OX3 antibody specific for rat Ia antigen was as strongly inhibitory as W3/25. It has previously been shown that W3/25 acts on the responder cell without being cytotoxic. Since OX3 antibody has been shown to bind to cells of $RT1^{l}$ rat strains but not to BN ($RT1^{n}$) or DA ($RT1^{av1}$) it may be presumed that the responder cell is also the target for the OX3HL inhibition. Neither W3/25 nor OX3 antibodies inhibited the polyclonal T cell response to PHA at 100 times the concentration at which the MLR was inhibited, suggesting that the mechanism of inhibition is specific for alloantigen stimulation. Since Ia antigen specific for OX3 antibody has not been previously detected on peripheral T cells these results suggest that the target for the inhibitory effect is either a very small subpopulation of T cells bearing Ia-associated receptors for stimulation or an Ia-bearing macrophage required for antigen presentation. Attempt to identify the functional subpopulation are underway.

ATTACHMENT NO. 4

Abstract: Fed. Proc. 40, 1033, 1981 - Presented at the FASEB meetings, Atlanta, Georgia, April, 1981

4553 IMMUNOLOGY

INHIBITION OF HUMAN PROLIFERATIVE RESPONSES TO ANTIGENS AND MITOGENS BY IRRADIATION AND ANTI DR ANTIBODIES. J.R.Lamb*, M.Brunswick*, E.Ketturer*, and J.N.Woody. Georgetown University School of Medicine, Washington, D.C. 20007

Dose response and kinetic studies were performed to define conditions under which optimal proliferative responses to influenza virus (IF), tetanus toxoid (TT) and pokeweed mitogen (PWM) could be obtained *in vitro* from human peripheral blood mononuclear leukocytes (PBM). For IF and TT separation of PBM into sheep erythrocyte (E) rosette positive (E^+) and negative (E^-) populations and irradiation prior to reconstitution revealed that the proliferative cell was E^+ but required the presence of an E^- cell to respond. Irradiation of E^+ cells in excess of 500R eliminated the response, whereas little effect was apparent even when the E^- population had been irradiated with 3000R prior to reconstitution. The PWM proliferative response was predominatly that of E^+ cells, however addition of E^- radiation resistant cells enhanced the response. The addition of rabbit anti-human Ia(p23,30) or a monoclonal anti-DR framework (DA2) antibodies in contrast to control antisera inhibited the proliferative response. These results suggest that the proliferative response to particulate (IF) and soluble (TT) antigens as well as mitogens require the presence of radiation sensitive E^+ cells as well as radiation resistant E^- cells. The inhibition of responses with anti-DR antibodies may suggest that essential cell interactions or factors are being blocked. (Supported by ONR contract N00014-77-C-0748).

ATTACHMENT NO. 5

ANTIGEN SPECIFIC HELPER FACTOR REACTS WITH ANTIBODIES TO HUMAN β_2 MICROGLOBULIN [1]

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1 This work was supported by a grant from the Medical Research Council, and in part by Office of Naval Research Contract N000-14-77-0748.

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5 Abbreviations used in this paper: MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; SAI/II, Streptococcus mutans antigen I/II complex; $MHF_{SAI/II}$, monkey helper factor specific for SAI/II; $HF_{SAI/II}$, mouse helper factor specific for SAI/II; $H\beta_2M$, human β_2-microglobulin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; KLH, keyhole limpet haemocyanin; DNP-SAI/II, dinitrophenylated SAI/II; Hepes, 4-(2-hydroxyethyl)-1 piperazine-ethane sulfonic acid; FCS, fetal calf serum; NaSCN, sodium thiocyanate; PBS, phosphate buffered saline; AFC, antibody forming cells; Fv, framework determinants of immunoglobulin variable region.

SUMMARY

Antigen specific helper factor was induced *in vitro* from lymphoid cells of monkeys and mice using an antigen derived from *Streptococcus mutans*. Helper activity was removed from supernatants of monkey cells by affinity chromatography on Sepharose 4B insolubilized antibodies specific for human β_2-microglobulin (Hβ_2M)[5] prepared in chicken, rabbit and rat. Also an insolublized monoclonal mouse anti-Hβ_2M antibody bound monkey helper factor activity. However, guinea pig antibody to human β_2M was inactive. In parallel studies, the pattern of absorption of mouse helper factor (HF) was different from the monkey, in that, insolubilized guinea pig anti-Hβ_2M bound helper factor, whereas rabbit and monoclonal anti-Hβ_2M failed to do so.

Although these findings are not compatible with an intact β_2M chain being present in helper factor they may imply a cross reactivity of β_2M with a "constant region" of helper factor which may share common sequences with β_2M. This may suggest that factor genes have evolved from the same ancestral genes as β_2M.

INTRODUCTION

The regulation of immune responses is mediated by a complex network of interacting cells, which either augment or suppress the overall response. Cell to cell contact does not appear to be an essential step for some pathways *in vitro*, and immunological regulation can be mediated by soluble factors which can be antigen specific (1-6) or non-specific (7-9) in nature, There is evidence that both these classes of factors may carry determinants encoded by genes mapping in the I region of the murine major histocompatibility complex (MHC), or in the analogous HLA-D region of the human MHC (10,11).

We have recently described an antigen specific helper factor (12) induced *in vitro* from the peripheral blood lymphocytes (PBL) of rhesus monkeys or from mouse spleen cells, upon stimulation with an antigen derived from the cariogenic bacterium *Streptococcus mutans* (SAI/II; 13,14). This helper factor had functional and biochemical properties identical to those described for specific helper factors which augment B cell responses to determinants on proteins or polypeptides (5,15). We report here the reaction of functionally active monkey ($MHF_{SAI/II}$) and mouse ($HF_{SAI/II}$) helper factor with purified anti-Hβ_2M antibody preparations raised in different species and a monoclonoal antibody that recognizes human β_2 microglobulin (Hβ_2M). The implications for factor structure are discussed.

MATERIALS AND METHODS

Animals: C57Bl/10.BR (B10.BR) mice bred at Guy's Hospital from breeding stock obtained from Imperial Cancer Research Fund Breeding Unit, Mill Hill, London and aged between three and six months were used for spleen cell cultures. Rhesus monkeys (*Macaca mulatta*) aged between two and three years and weighing 2 to 3.5 kg. bled from the femoral vessels provided a source of PBL's.

Antigens: SAI/II was prepared from the culture supernatant of *Streptococcus mutans* (Serotype c; Guy's strain) as described in detail (13,14). Briefly, an ammonium sulphate precipitate was collected, redissolved and separated on a DEAE cellulose column. The eluted antigen was further purified on Sepharose 6B (Pharmacia, Uppsala, Sweden) and characterized as showing a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The antigen consisted predominantly of protein with an apparent molecular weight of 185,000 daltons. Keyhole limpet haemocyanin (KLH) was the gift of Dr. M. Rittenberg, Portland, Oregon. Dinitrophenylated SAI/II (DNP-SAI/II) prepared as previously described (12) using dinitrofluorobenzene had five groups of DNP/100,000 daltons.

Tissue Culture Media: Cell suspensions were cultured in Hepes (4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid) buffered RPMI 1640 supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml) and 5% fetal calf serum (FCS; Gibco, Paisley, Scotland). The outer compartment of Marbrook-Diener tissue culture flasks and Costar plates (Bellco Glass, New Jersey) were filled with bicarbonate buffered RPMI 1640, supplemented with 5% FCS and 5×10^{-5}M 2-mercaptoethanol.

Antisera: Chicken, rabbit, rat, and guinea pig anti-Hβ_2M antisera were prepared by immunizing groups of animals (50-200 μg/kg) intramuscularly with Hβ_2M in complete Freund's adjuvant followed by three weekly boosts in

incomplete Freund's adjuvant until a strong response was detected by radioimmunoassay (16), with Hβ_2M purified from the urine of patients with Willson's disease (17). Monoclonal anti-Hβ_2M was prepared from a hybridoma culture kindly supplied by Ceppellini and Trucco (18) which was produced by fusing BALB/C spleen cells previously immunized with human PBLs with the plasmacytoma as described by Kohler and Milstein (19). Positive clones were identified by the binding of supernatant immunoglobulin to human lymphocytes but not to human erythrocytes. Confirmation of anti-Hβ_2M activity was obtained by the direct binding of supernatant immunoglobulin to wells of microtitre trays coated with human β_2M (Sanderson, unpublished). Immunoglobulin having anti-Hβ_2M activity was purified from sera by absorption and elution from Hβ_2M columns consisting of the pure protein bound to activated sepharose 4B (Pharmacia, Uppsala, Sweden), using 3M sodium thiocyanate (NaSCN) or 0.5% acetic acid as eluent. Purity of Hβ_2M or antibody was confirmed by SDS-PAGE and isoelectric focussing.

Preparation and use of immunoadsorbents

The antigens, SAI/II and KLH, and the anti-H M antisera were coupled to cyanogen bromide activated sepharose 4B. The purified proteins were coupled at 1mg per ml of adsorbent. Before use the immunoadsorbents were washed once with 3M NaSCN followed by three times with phosphate buffered saline (PBS). For absorption, factors at a dilution of 10^{-1} were used in the ratio of 1ml of factor to 1ml of beads. After mixing for two hours at 4°C the unbound material was recovered by centrifugation, millipore filtered and stored at -20°C until used. The beads were washed five times with PBS and the bound material eluted from the beads by washing with 3M NaSCN in the ratio of 1ml per ml of beads. The eluted material was diluted with an equal volume of PBS, dialyzed against saline for 24 hours, millipore filtered and stored at -20°C. The absorptions were carried out on more than one occasion using the

same batch of helper factor. In addition other absorptions were performed with a fewer number of immunoadsorbent preparations, using four different batches of helper factor. The pattern of reactivity was consistent within each species.

The preparation and assay of helper factors

The preparation and assay of the helper factors was performed as previously described (12). Briefly, mouse (B10.BR) spleen cells or monkey PBL at $15x10^6$ and $5x10^6$/ml respectively were primed *in vitro* in Marbrook-Diener flasks with 0.01μg/ml of SAI/II for four days to induce helper cells. These cells were harvested, washed and restimulated with 0.1μg/ml of SAI/II for 24 hours and the cell free supernatants (helper factor; $HF_{SAI/II}$ and $MHF_{SAI/II}$) were collected. $HF_{SAI/II}$ and $MHF_{SAI/II}$ at a final concentration of 10^{-3}, the previously determined optimum concentration for all batches tested so far were assayed in Marbrook-Diener flasks or Costar plates in the presence of 10^7 or $5x10^6$ unprimed (input) B10.BR spleen cells, respectively, and 0.1μg/ml of DNP-SAI/II (12). The anti-DNP antibody forming cells (AFC) were assayed on day 4, using the modified Cunningham assay (19) using DNP-Fab coated SRC and uncoated SRC. DNP specific plaques were the difference between the two. Since unprimed spleen cells were used, only IgM AFC were detected. All cultures were carried out in triplicate and assayed separately. Within each experiment the numbers of AFC in each group were compared to a background of the response of 10^7 or $5x10^6$ unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II.

RESULTS

Antigenic Specificity of Helper Factor: Immunoadsorbents were used as probes to examine the antigenic specificity of helper factors used prior to their further characterization. The activity of monkey and mouse helper

factors induced with the antigen SAI/II bound to immunoabsorbent columns of SAI/II and were eluted with NaSCN. However, the activity failed to bind to columns of the unrelated protein antigen KLH (Table 1).

Reaction of helper factor with insolubilized anti-Hβ2)-microglobulin antibody

The reaction of MHF with immunoadsorbents comprising of affinity purified heterologous antibodies to Hβ_2M, and a similarly purified monoclonal antibody to Hβ_2M are shown in Figure 1. The activity of MHF could be absorbed by and eluted from the chicken, rabbit, and rat as well as mouse monoclonal anti-Hβ_2M columns using 3M-NaSCN. In addition, it has been found that MHF could be competitively eluted from monoclonal anti-Hβ_2M columns with pure β_2M (Zanders, Lamb and Sanderson, unpublished observation). Guinea pig anti-Hβ_2M immunoadsorbents did not react with determinants present in monkey helper factor.

Analogous experiments were performed using SAI/II specific helper factor from B10.BR mice (Fig. 2). The experiments revealed that anti-Hβ_2M likewise bound helper activity, but the pattern of absorption was different from that obtained when monkey factor was reacted with the same immunoadsorbents. The functional activity of mouse helper factor was absorbed by columns containing chicken, rat and guinea pig anti-Hβ_2M antibodies. The latter is contrary to that observed with monkey factor; similarily the inability of monoclonal anti-Hβ_2M to absorb mouse helper factor. The rabbit anti-Hβ_2M antibody also failed to bind the activity of mouse $HF_{SAI/II}$.

DISCUSSION

Antigen specific factors have been reported to react with antisera specific for immunoglobulin variable region idiotypes (11,21) and framework

(Fv) structures (22). Furthermore, antisera directed against factors have been reported which recognize determinants linked to the functional type of factor (e.g. helper or suppressor) but not to determinants related to the specificity or mouse strain of origin of the factor (23). These sites of cross reactivity were termed "constant regions" (23). Neither HLA (heavy chain) determinants in man (Lamb, Zanders and Sanderson, unpublished observation), nor those of H-2K or D regions in the mouse (5,24) have been found on factors.

The results presented here demonstrate absorption of the activity of monkey antigen specific helper factor by chicken, rabbit, rat and monoclonal (mouse) anti-Hβ_2M antibody, whereas guinea pig anti-Hβ_2M did not recognize determinants in monkey helper factor. The absorption capacity of the columns was high since 1mg of purified anti-Hβ_2M was used to adsorb 1ml of helper factor diluted 10^{-1} in all cases and, thus the failure of guinea pig anti-Hβ_2M to absorb monkey HF is not likely to be due to inadequate absorption capacity. This was verified by the fact that the column absorbed all of the mouse HF (Fig. 2).

Mouse helper factor also reacted with anti-Hβ_2M antibodies, but with a different pattern of binding as compared to monkey helper factor. Mouse helper factor bound to chicken, rat and guinea pig anti-Hβ_2M but not to rabbit or monoclonal anti-Hβ_2M antibody. The activity of the latter reagents were verified by their capacity to react with monkey helper factor.

There are several possible explanations for the failure of guinea pig anti-humanβ_2M to bind monkey factor or for rabbit anti-humanβ_2M to bind mouse factor. The amino acid sequences recognized in humanβ_2M by guinea pigs may not occur in monkey, if they do occur then they may be obscured by other protein chains in the total moiety which comprises active helper factor. Indeed tertiary structural features within the β_2M like molecule occurring in

helper factor may similarly explain the failure of guinea pig anti human β_2M to behave as other mammalian anti-human β_2M reagents do. It has also been found that a determinant on free human β_2M cannot be detected in the intact HLA molecule. (A. R. Sanderson, personal communication). Similar considerations apply to the rabbit anti-human β_2M to bind mouse factors. The case of failure of monoclonal mouse anti-human β_2M to bind mouse helper factor however is different. No mouse immune reagent is likely to bind to a mouse β_2M component in mouse helper factor because β_2M is monomorphic within all species even in mouse where it has been claimed to be dimorphic (25) the dimorphism is not across the mouse strain differences used in this study (Balb/C for monoclonal reagents; B10BR for factors).

The results are reminiscent of observations on allogeneic effect factor (26), although the serological evidence for the presence of β_2M determinants was less unequivocal in this earlier study, since whole anti-β_2M antisera were used, and absorption data with purified antibody was not reported. It remains to be determined whether β_2M cross reactivity is a property shared also by helper factors which lack antigen specificity in addition to the antigen specific factors reported here. Furthermore, a factor reactive with anti-β_2M antibodies has been reported that specificity suppressed IgE antibody production (27). The antigenic similarity between β_2M and helper factor led us to test the possibility that intact Hβ_2M may directly stimulate antibody production by mouse spleen cells either alone or in the presence of specific antigen. However, no effect was found upon adding Hβ_2M at concentrations from 0.01-1.0μg/ml. (Data not shown.)

There are a number of possible interpretations which can be drawn from our data. The different patterns of binding between the two factors imply that an intact β_2M chain is probably not present in factors, since it would have been expected that all sera to Hβ_2M would bind monkey helper factor. Although

there is no precedent for the production of β_2M chain fragments, it is possible that helper factor contains a portion of the β_2M chain, or that a polypeptide region is shared by β_2M and helper factor. The latter interpretation appears to be more likely in view of the evidence that β_2M shows sequence homology with the C_H3 domain of IgG and serological cross reactivity suggests that helper factor contains a region analogous to an immunoglobulin C_H domain. This concept is compatible with evidence that some factors bind to protein A (M. Cecka and R. Cone, personal communication) and with certain anti-Ig reagents especially anti-IgM (1,5,15).

Factors contain a variable region which is "immunoglobulin-like" bearing Ig idiotype and Ig framework (Fv) determinants (22). Since anti-Hβ_2M does not react with immunoglobulin (Sanderson, unpublished observation), it is unlikely that the anti-Hβ_2M antibodies are reacting with the variable region of helper factor. Thus the reaction is presumably with the "constant region" of factors (23) and this reactivity with anti-Hβ_2M may thus be a reflection that the origin of the genes controlling factor "constant regions" may be products of the same ancestral genes that gave rise to β_2M.

Clearly further work is necessary to clarify the precise nature and function of these regulatory factors. Meanwhile reagents based on monoclonal antibodies having defined specificity offer considerble promise in purification procedures.

ACKNOWLEDGEMENTS

The technical assistance of Janet Avery and Carol Robinson is gratefully acknowledged. The authors wish to thank Dr. M. Rittenberg for the gift of antigen and Dr. T. Kindt for his critical review of this manuscript.

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Legend to Table 1

* Mouse ($HF_{SAI/II}$) and monkey ($MHF_{SAI/II}$) helper factor induced with SAI/II were cultured at a final concentration of 10^{-3} with 10^7 unprimed B10.BR spleen cells in the presence of 0.1μg of DNP-SAI/II for four days.

** Background response of 10^7 unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II without added helper factor.

*** Response of $HF_{SAI/II}$ and $MHF_{SAI/II}$ cultured with 10^7 unprimed B10.BR spleen cells without added DNP-SAI/II.

Students t test was used to assess the significance of treated as compared to untreated helper factor. n=6, A,$P<0.05$; B,$p<0.01$; C,$<$ p 0.001.

Six other experiments gave similar results. None yielded contrary findings.

TABLE 1

Antigenic Specificity of Mouse and Monkey Helper Factor

STIMULUS*			RESPONSE
HF	Antigen	Immunoadsorbent	Anti-DNP AFC/culture ± SE
	DNP-SAI/II		47±12**
$HF_{SAI/II}$	-	-	30±6***
+	+	-	470±57
+	+	KLH bound	80±17[C]
+	+	+ unbound	350±70
+	+	SAI/II bound	410±45
+	+	+ unbound	27±12[C]
$MHF_{SAI/II}$	-	-	60±21***
+	+	-	640±21
+	+	KLH bound	97±17[C]
+	+	+ unbound	480±71[A]
+	+	SAI/II bound	527±41[C]
+	+	+ unbound	107±20[C]

TABLE 1

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+	+	KLH bound	80±17[C]
+	+	+ unbound	350±70
+	+	SAI/II bound	410±45
+	+	+ unbound	27±12[C]
$MHF_{SAI/II}$	-	-	60±21***
+	+	-	640±21
+	+	KLH bound	97±17[C]
+	+	+ unbound	480±71[A]
+	+	SAI/II bound	527±41[C]
+	+	+ unbound	107±20[C]

Legend to Figures

Figure 1. Reaction of monkey helper factor with anti-human β_2 microglobulin antisera.

Monkey helper factor ($MHF_{SAI/II}$) induced with SAI/II was added at a final concentration of 10^{-3} to 10^7 unprimed B10.BR spleen cells in presence of 0.1μg/ml of DNP-SAI/II. Cultures were performed in Marbrook flasks. Anti-DNP AFC were assayed on day 4.

$MHF_{SAI/II}$ was bound and eluted (3M-NaSCN) from Sepharose 4B columns anti-Hβ_2M from which antisera had been insolubilized at 1mg/ml.

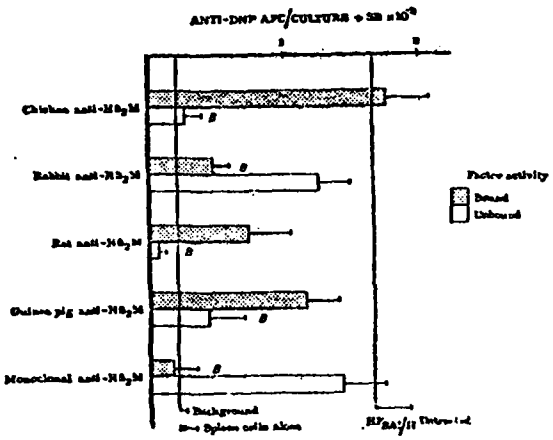
Background is the response of 10^7 unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II without added helper factor. Students t test was used to assess the significance of treated as compard to untreated $MHF_{SAI/II}$. n=6, A, $P<0.05$ B, $P<0.01$; C, $P<0.001$.

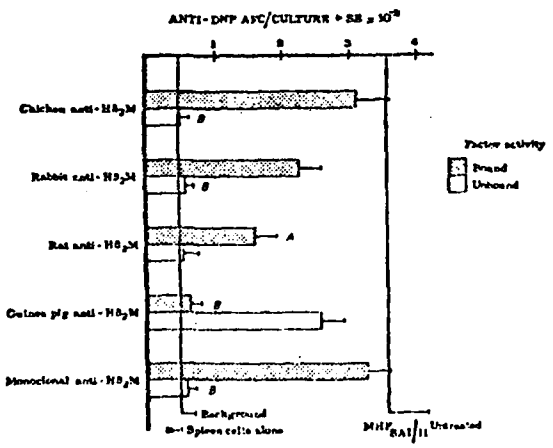
Two other experiments gave similar results.

Figure 2. Reaction of mouse helper factor with anti-human $_2$ microglobulin antisera.

Mouse helper factor ($HF_{SAI/II}$) was assayed at 10^{-3} final concentration on $5x10^6$ unprimed (input) B10.BR spleen cells in Costar plates in the presence of 0.1μg/ml of DNP-SAI/II.

For absorptions, background and p values, see legend to Figure 1.





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ATTACHMENT NO. 5

ANTIGEN SPECIFIC HELPER FACTOR REACTS WITH ANTIBODIES TO HUMAN β_2 MICROGLOBULIN [1]

1 This work was supported by a grant from the Medical Research Council, and in part by Office of Naval Research Contract N000-14-77-0748.

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5 Abbreviations used in this paper: MHC, major histocompatibility complex; PBL, peripheral blood lymphocytes; SAI/II, Streptococcus mutans antigen I/II complex; $MHF_{SAI/II}$, monkey helper factor specific for SAI/II; $HF_{SAI/II}$, mouse helper factor specific for SAI/II; $H\beta_2M$, human β_2-microglobulin; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; KLH, keyhole limpet haemocyanin; DNP-SAI/II, dinitrophenylated SAI/II; Hepes, 4-(2-hydroxyethyl)-1 piperazine-ethane sulfonic acid; FCS, fetal calf serum; NaSCN, sodium thiocyanate; PBS, phosphate buffered saline; AFC, antibody forming cells; Fv, framework determinants of immunoglobulin variable region.

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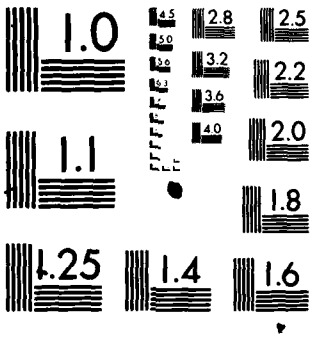

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SUMMARY

Antigen specific helper factor was induced *in vitro* from lymphoid cells of monkeys and mice using an antigen derived from *Streptococcus mutans*. Helper activity was removed from supernatants of monkey cells by affinity chromatography on Sepharose 4B insolubilized antibodies specific for human β_2-microglobulin ($H\beta_2M$)[5] prepared in chicken, rabbit and rat. Also an insolublized monoclonal mouse anti-$H\beta_2M$ antibody bound monkey helper factor activity. However, guinea pig antibody to human β_2M was inactive. In parallel studies, the pattern of absorption of mouse helper factor (HF) was different from the monkey, in that, insolubilized guinea pig anti-$H\beta_2M$ bound helper factor, whereas rabbit and monoclonal anti-$H\beta_2M$ failed to do so.

Although these findings are not compatible with an intact β_2M chain being present in helper factor they may imply a cross reactivity of β_2M with a "constant region" of helper factor which may share common sequences with β_2M. This may suggest that factor genes have evolved from the same ancestral genes as β_2M.

INTRODUCTION

The regulation of immune responses is mediated by a complex network of interacting cells, which either augment or suppress the overall response. Cell to cell contact does not appear to be an essential step for some pathways *in vitro*, and immunological regulation can be mediated by soluble factors which can be antigen specific (1-6) or non-specific (7-9) in nature, There is evidence that both these classes of factors may carry determinants encoded by genes mapping in the I region of the murine major histocompatibility complex (MHC), or in the analogous HLA-D region of the human MHC (10,11).

We have recently described an antigen specific helper factor (12) induced *in vitro* from the peripheral blood lymphocytes (PBL) of rhesus monkeys or from mouse spleen cells, upon stimulation with an antigen derived from the cariogenic bacterium *Streptococcus mutans* (SAI/II; 13,14). This helper factor had functional and biochemical properties identical to those described for specific helper factors which augment B cell responses to determinants on proteins or polypeptides (5,15). We report here the reaction of functionally active monkey ($MHF_{SAI/II}$) and mouse ($HF_{SAI/II}$) helper factor with purified anti-Hβ_2M antibody preparations raised in different species and a monoclonoal antibody that recognizes human β_2 microglobulin (Hβ_2M). The implications for factor structure are discussed.

MATERIALS AND METHODS

Animals: C57Bl/10.BR (B10.BR) mice bred at Guy's Hospital from breeding stock obtained from Imperial Cancer Research Fund Breeding Unit, Mill Hill, London and aged between three and six months were used for spleen cell cultures. Rhesus monkeys (*Macaca mulatta*) aged between two and three years and weighing 2 to 3.5 kg. bled from the femoral vessels provided a source of PBL's.

Antigens: SAI/II was prepared from the culture supernatant of *Streptococcus mutans* (Serotype c; Guy's strain) as described in detail (13,14). Briefly, an ammonium sulphate precipitate was collected, redissolved and separated on a DEAE cellulose column. The eluted antigen was further purified on Sepharose 6B (Pharmacia, Uppsala, Sweden) and characterized as showing a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The antigen consisted predominantly of protein with an apparent molecular weight of 185,000 daltons. Keyhole limpet haemocyanin (KLH) was the gift of Dr. M. Rittenberg, Portland, Oregon. Dinitrophenylated SAI/II (DNP-SAI/II) prepared as previously described (12) using dinitrofluorobenzene had five groups of DNP/100,000 daltons.

Tissue Culture Media: Cell suspensions were cultured in Hepes (4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid) buffered RPMI 1640 supplemented with penicillin (100 IU/ml), streptomycin (100 IU/ml) and 5% fetal calf serum (FCS; Gibco, Paisley, Scotland). The outer compartment of Marbrook-Diener tissue culture flasks and Costar plates (Bellco Glass, New Jersey) were filled with bicarbonate buffered RPMI 1640, supplemented with 5% FCS and 5×10^{-5}M 2-mercaptoethanol.

Antisera: Chicken, rabbit, rat, and guinea pig anti-Hβ_2M antisera were prepared by immunizing groups of animals (50-200 μg/kg) intramuscularly with Hβ_2M in complete Freund's adjuvant followed by three weekly boosts in

incomplete Freund's adjuvant until a strong response was detected by radioimmunoassay (16), with Hβ_2M purified from the urine of patients with Wilson's disease (17). Monoclonal anti-Hβ_2M was prepared from a hybridoma culture kindly supplied by Ceppellini and Trucco (18) which was produced by fusing BALB/C spleen cells previously immunized with human PBLs with the plasmacytoma as described by Kohler and Milstein (19). Positive clones were identified by the binding of supernatant immunoglobulin to human lymphocytes but not to human erythrocytes. Confirmation of anti-Hβ_2M activity was obtained by the direct binding of supernatant immunoglobulin to wells of microtitre trays coated with human β_2M (Sanderson, unpublished). Immunoglobulin having anti-Hβ_2M activity was purified from sera by absorption and elution from Hβ_2M columns consisting of the pure protein bound to activated sepharose 4B (Pharmacia, Uppsala, Sweden), using 3M sodium thiocyanate (NaSCN) or 0.5% acetic acid as eluent. Purity of Hβ_2M or antibody was confirmed by SDS-PAGE and isoelectric focussing.

Preparation and use of immunoadsorbents

The antigens, SAI/II and KLH, and the anti-H M antisera were coupled to cyanogen bromide activated sepharose 4B. The purified proteins were coupled at 1mg per ml of adsorbent. Before use the immunoadsorbents were washed once with 3M NaSCN followed by three times with phosphate buffered saline (PBS). For absorption, factors at a dilution of 10^{-1} were used in the ratio of 1ml of factor to 1ml of beads. After mixing for two hours at 4°C the unbound material was recovered by centrifugation, millipore filtered and stored at -20°C until used. The beads were washed five times with PBS and the bound material eluted from the beads by washing with 3M NaSCN in the ratio of 1ml per ml of beads. The eluted material was diluted with an equal volume of PBS, dialyzed against saline for 24 hours, millipore filtered and stored at -20°C. The absorptions were carried out on more than one occasion using the

same batch of helper factor. In addition other absorptions were performed with a fewer number of immunoadsorbent preparations, using four different batches of helper factor. The pattern of reactivity was consistent within each species.

The preparation and assay of helper factors

The preparation and assay of the helper factors was performed as previously described (12). Briefly, mouse (B10.BR) spleen cells or monkey PBL at 15×10^6 and 5×10^6/ml respectively were primed *in vitro* in Marbrook-Diener flasks with 0.01μg/ml of SAI/II for four days to induce helper cells. These cells were harvested, washed and restimulated with 0.1μg/ml of SAI/II for 24 hours and the cell free supernatants (helper factor; $HF_{SAI/II}$ and $MHF_{SAI/II}$) were collected. $HF_{SAI/II}$ and $MHF_{SAI/II}$ at a final concentration of 10^{-3}, the previously determined optimum concentration for all batches tested so far were assayed in Marbrook-Diener flasks or Costar plates in the presence of 10^7 or 5×10^6 unprimed (input) B10.BR spleen cells, respectively, and 0.1μg/ml of DNP-SAI/II (12). The anti-DNP antibody forming cells (AFC) were assayed on day 4, using the modified Cunningham assay (19) using DNP-Fab coated SRC and uncoated SRC. DNP specific plaques were the difference between the two. Since unprimed spleen cells were used, only IgM AFC were detected. All cultures were carried out in triplicate and assayed separately. Within each experiment the numbers of AFC in each group were compared to a background of the response of 10^7 or 5×10^6 unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II.

RESULTS

Antigenic Specificity of Helper Factor: Immunoadsorbents were used as probes to examine the antigenic specificity of helper factors used prior to their further characterization. The activity of monkey and mouse helper

factors induced with the antigen SAI/II bound to immunoabsorbent columns of SAI/II and were eluted with NaSCN. However, the activity failed to bind to columns of the unrelated protein antigen KLH (Table 1).

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Antigen specific factors have been reported to react with antisera specific for immunoglobulin variable region idiotypes (11,21) and framework

(Fv) structures (22). Furthermore, antisera directed against factors have been reported which recognize determinants linked to the functional type of factor (e.g. helper or suppressor) but not to determinants related to the specificity or mouse strain of origin of the factor (23). These sites of cross reactivity were termed "constant regions" (23). Neither HLA (heavy chain) determinants in man (Lamb, Zanders and Sanderson, unpublished observation), nor those of H-2K or D regions in the mouse (5,24) have been found on factors.

The results presented here demonstrate absorption of the activity of monkey antigen specific helper factor by chicken, rabbit, rat and monoclonal (mouse) anti-Hβ_2M antibody, whereas guinea pig anti-Hβ_2M did not recognize determinants in monkey helper factor. The absorption capacity of the columns was high since 1mg of purified anti-Hβ_2M was used to adsorb 1ml of helper factor diluted 10^{-1} in all cases and, thus the failure of guinea pig anti-Hβ_2M to absorb monkey HF is not likely to be due to inadequate absorption capacity. This was verified by the fact that the column absorbed all of the mouse HF (Fig. 2).

Mouse helper factor also reacted with anti-Hβ_2M antibodies, but with a different pattern of binding as compared to monkey helper factor. Mouse helper factor bound to chicken, rat and guinea pig anti-Hβ_2M but not to rabbit or monoclonal anti-Hβ_2M antibody. The activity of the latter reagents were verified by their capacity to react with monkey helper factor.

There are several possible explanations for the failure of guinea pig anti-humanβ_2M to bind monkey factor or for rabbit anti-human β_2M to bind mouse factor. The amino acid sequences recognized in humanβ_2M by guinea pigs may not occur in monkey, if they do occur then they may be obscured by other protein chains in the total moiety which comprises active helper factor. Indeed tertiary structural features within the β_2M like molecule occurring in

helper factor may similarly explain the failure of guinea pig anti human β_2M to behave as other mammalian anti-human β_2M reagents do. It has also been found that a determinant on free human β_2M cannot be detected in the intact HLA molecule. (A. R. Sanderson, personal communication). Similar considerations apply to the rabbit anti-human β_2M to bind mouse factors. The case of failure of monoclonal mouse anti-human β_2M to bind mouse helper factor however is different. No mouse immune reagent is likely to bind to a mouse β_2M component in mouse helper factor because β_2M is monomorphic within all species even in mouse where it has been claimed to be dimorphic, (25) the dimorphism is not across the mouse strain differences used in this study (Balb/C for monoclonal reagents; B10BR for factors).

The results are reminiscent of observations on allogeneic effect factor (26), although the serological evidence for the presence of β_2M determinants was less unequivocal in this earlier study, since whole anti-β_2M antisera were used, and absorption data with purified antibody was not reported. It remains to be determined whether β_2M cross reactivity is a property shared also by helper factors which lack antigen specificity in addition to the antigen specific factors reported here. Furthermore, a factor reactive with anti-β_2M antibodies has been reported that specificity suppressed IgE antibody production (27). The antigenic similarity between β_2M and helper factor led us to test the possibility that intact Hβ_2M may directly stimulate antibody production by mouse spleen cells either alone or in the presence of specific antigen. However, no effect was found upon adding Hβ_2M at concentrations from 0.01-1.0 μg/ml. (Data not shown.)

There are a number of possible interpretations which can be drawn from our data. The different patterns of binding between the two factors imply that an intact β_2M chain is probably not present in factors, since it would have been expected that all sera to Hβ_2M would bind monkey helper factor. Although

there is no precedent for the production of β_2M chain fragments, it is possible that helper factor contains a portion of the β_2M chain, or that a polypeptide region is shared by β_2M and helper factor. The latter interpretation appears to be more likely in view of the evidence that β_2M shows sequence homology with the C_H3 domain of IgG and serological cross reactivity suggests that helper factor contains a region analogous to an immunoglobulin C_H domain. This concept is compatible with evidence that some factors bind to protein A (M. Cecka and R. Cone, personal communication) and with certain anti-Ig reagents especially anti-IgM (1,5,15).

Factors contain a variable region which is "immunoglobulin-like" bearing Ig idiotype and Ig framework (Fv) determinants (22). Since anti-Hβ_2M does not react with immunoglobulin (Sanderson, unpublished observation), it is unlikely that the anti-Hβ_2M antibodies are reacting with the variable region of helper factor. Thus the reaction is presumably with the "constant region" of factors (23) and this reactivity with anti-Hβ_2M may thus be a reflection that the origin of the genes controlling factor "constant regions" may be products of the same ancestral genes that gave rise to β_2M.

Clearly further work is necessary to clarify the precise nature and function of these regulatory factors. Meanwhile reagents based on monoclonal antibodies having defined specificity offer considerble promise in purification procedures.

ACKNOWLEDGEMENTS

The technical assistance of Janet Avery and Carol Robinson is gratefully acknowledged. The authors wish to thank Dr. M. Rittenberg for the gift of antigen and Dr. T. Kindt for his critical review of this manuscript.

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Legend to Table 1

* Mouse ($HF_{SAI/II}$) and monkey ($MHF_{SAI/II}$) helper factor induced with SAI/II were cultured at a final concentration of 10^{-3} with 10^7 unprimed B10.BR spleen cells in the presence of 0.1μg of DNP-SAI/II for four days.

** Background response of 10^7 unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II without added helper factor.

*** Response of $HF_{SAI/II}$ and $MHF_{SAI/II}$ cultured with 10^7 unprimed B10.BR spleen cells without added DNP-SAI/II.

Students t test was used to assess the significance of treated as compared to untreated helper factor. n=6, A,$P<0.05$; B,$p<0.01$; C,$<$ p 0.001.

Six other experiments gave similar results. None yielded contrary findings.

TABLE 1

Antigenic Specificity of Mouse and Monkey Helper Factor

HF	STIMULUS* Antigen	Immunoadsorbent	RESPONSE Anti-DNP AFC/culture ± SE
	DNP-SAI/II		47±12**
$HF_{SAI/II}$	-	-	30±6***
+	+	-	470±57
+	+	KLH bound	80±17[C]
+	+	+ unbound	350±70
+	+	SAI/II bound	410±45
+	+	+ unbound	27±12[C]
$MHF_{SAI/II}$	-	-	60±21***
+	+	-	640±21
+	+	KLH bound	97±17[C]
+	+	+ unbound	480±71[A]
+	+	SAI/II bound	527±41[C]
+	+	+ unbound	107±20[C]

Legend to Figures

Figure 1. Reaction of monkey helper factor with anti-human β_2 microglobulin antisera.

Monkey helper factor ($MHF_{SAI/II}$) induced with SAI/II was added at a final concentration of 10^{-3} to 10^7 unprimed B10.BR spleen cells in presence of 0.1μg/ml of DNP-SAI/II. Cultures were performed in Marbrook flasks. Anti-DNP AFC were assayed on day 4.

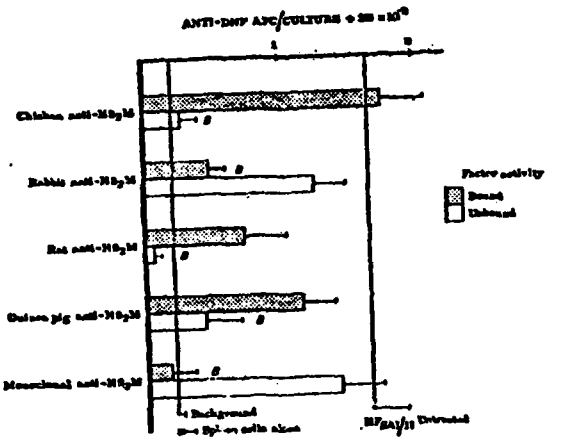
$MHF_{SAI/II}$ was bound and eluted (3M-NaSCN) from Sepharose 4B columns anti-Hβ_2M from which antisera had been insolubilized at 1mg/ml. Background is the response of 10^7 unprimed B10.BR spleen cells to 0.1μg/ml of DNP-SAI/II without added helper factor. Students t test was used to assess the significance of treated as compard to untreated $MHF_{SAI/II}$. n=6, A, $P<0.05$ B, $P<0.01$; C, $P<0.001$.

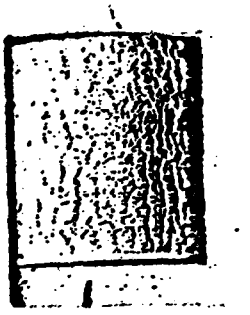
Two other experiments gave similar results.

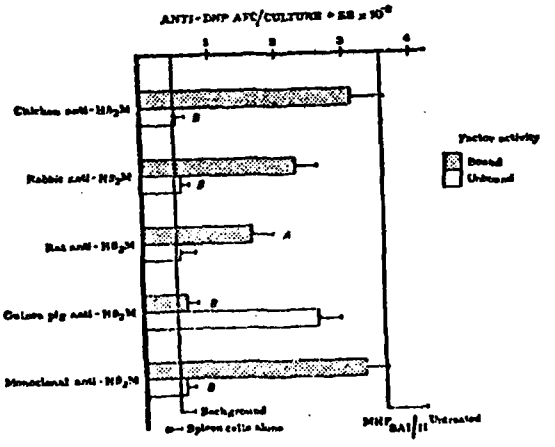
Figure 2. Reaction of mouse helper factor with anti-human $_2$ microglobulin antisera.

Mouse helper factor ($HF_{SAI/II}$) was assayed at 10^{-3} final concentration on $5x10^6$ unprimed (input) B10.BR spleen cells in Costar plates in the presence of 0.1μg/ml of DNP-SAI/II.

For absorptions, background and p values, see legend to Figure 1.







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